Ion Speciation in Milk-like Systems

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This research was conducted under the auspices of the Graduate School of Voeding, Levensmiddelentechnologie, Agrobiotechnologie en Gezondheid (VLAG).

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Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. ir. M. J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 29 September 2010
at 4 p.m. in the Aula

Ran Gao
Ion Speciation in Milk-like Systems,
250 pages.
PhD Thesis, Wageningen University, Wageningen, The Netherlands (2010)

ISBN: 978-90-8585-722-8

Dedicated to my parents and my wife

Abstract

The research described in this thesis is about ion behaviour in milk systems as a function of changing conditions (pH, ionic strength, addition of electrolytes and non-electrolytes). The focus was on (i) quantification of the ion equilibria in milk systems based on experimental conditions, (ii) development of quantitative models describing the ion equilibria. The so-called Donnan Membrane Technique (DMT) was successfully applied to determine the concentrations of free Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} ions in milk-like systems. Next, models were developed to describe ion equilibria in simulated milk ultrafiltrate solution (SMUF) as a first step to build models for milk systems. Freshly prepared SMUF appeared not to be in thermodynamic equilibrium regarding the supersaturation of calcium phosphate. Therefore, the equilibrium ion speciation (EIS) model and the dynamic ion speciation (DIS) model were developed for SMUF at equilibrium and at initial state, respectively. Both EIS and DIS models gave satisfactory predictions in ion equilibria in SMUF enriched with different electrolytes. Also, addition of non-electrolytes such as various disaccharides appeared to remarkably increase the Ca^{2+} and H^{+} ion activities in SMUF and milk. The observed results could be explained by a theory called Mean Spherical Approximation (MSA), which suggests that excluded volume effects due to addition of sugars account for the increase in Ca^{2+} and H^{+} ion activity coefficients and thus on Ca^{2+} and H^{+} ion activities. Based on the EIS and DIS models, a model named Milk Ion Speciation (MIS) was built to describe ion equilibria in milk. The MIS model was able to predict the ion composition in milk and milk enriched with different electrolytes. In addition, the methodologies of Ca^{2+} activity measurement were reviewed. We found that the calcium ion selective electrode (Ca-ISE) underestimated the Ca^{2+} activity at ionic strength lower

than 0.1 mol/kg water compared to DMT. It appeared that the ionic composition of conventional calibration standards significantly affects the Ca ion activity measurement. A series of new calibration standards, having similar composition to milk serum, was developed and this remarkably improved the Ca^{2+} activity measurement in milk-like systems. The developed ion speciation models were carefully evaluated. Particularly, the current limitations of the models such as the nature of CCP and the effect of adding polyphosphate were intensively discussed. Moreover, the models were tested to calculate ion composition in cheese. It turns out that the models offer great potential to correlate ion properties with the functional properties of dairy products.

Table of Contents

Chapter 1

General Introduction

1.1 Ion equilibria

This thesis is about the ion behaviours in milk and systems derived from milk. The mineral fraction of milk is relatively small (approximately 8-9 g L^{-1}) (Gaucheron, 2005). Potassium, sodium, calcium and magnesium ions are the main cations, and phosphate, citrate and chloride are the dominant anions. Milk minerals are partitioned between the colloidal and aqueous phases. Approximately 70% of calcium, 30% of magnesium, 50% of phosphate and 10% of citrate are present as undissolved complexes in casein micelles, named colloidal calcium phosphate (CCP) (Holt, 1997). In the milk serum phase, salts exist either as free ions or as ion pairs/complexes. Sodium and potassium ions form only weak ion pairs with chloride, citrate and phosphate and therefore mainly remain as free ions. The divalent cations (Ca^{2+} and Mg^{2+}) are mainly complexed with citrate and to a lesser extent with HPO_4^{2-}. There is a dynamic and very rapidly attained equilibrium between the ion pairs and the free ions in the aqueous phase. Moreover, there is a dynamic but slowly attained equilibrium between salts in the aqueous phase and colloidal phase (Walstra & Jenness, 1984). The ion equilibria are known to play an important role in the structure and stability of casein micelles (Horne, 1998; Walstra, 1990). Alterations of the ion equilibria induce significant changes in the ionic composition between the colloidal phase and aqueous phase. This will have effects on the physicochemical properties of casein micelles and the stability of products during processing and storage (De La Fuente, 1998; Fox & McSweeney, 1998; Huppertz & Fox, 2006). Therefore, it is of great importance to understand the ion equilibria under practical conditions.

1.2 Ion equilibria – thermodynamic considerations

The properties of a salt solution, such as the state of ionization of the various components, are not governed by concentrations but by activities (Walstra &

Jenness, 1984). For a given ion species i, the general relationship between activity a_i and (molality) concentration m_i is written as follows:

$$a_i = m_i \cdot \gamma_i \quad (1)$$

where γ_i is the molal activity coefficient. The same form of the equation is valid for molar concentrations but it should be realized that the activity coefficient depends on the unit chosen; in this thesis we will use the symbol y_i for the molar activity coefficient to underline this dependency.

As described in section 1.1, ions in milk systems are present in several forms, such as free ions, ion pairs/complexes and undissolved compounds. In other words, there are many association and dissociation reactions ongoing in milk, resulting in formation of dynamic ion equilibria. All the reaction rates of the ions depend on their activities. For example, the reaction for $CaCl^+$ ion pair formation is given by:

$$Ca^{2+} + Cl^- \leftrightarrow CaCl^+ \quad (2)$$

For the association equilibrium of $CaCl^+$, we have an association constant:

$$K_{CaCl^+} = \frac{a_{CaCl^+}}{a_{Ca^{2+}} \cdot a_{Cl^-}} = \frac{m_{CaCl^+}}{m_{Ca^{2+}} \cdot m_{Cl^-}} \cdot \frac{\gamma_{CaCl^+}}{\gamma_{Ca^{2+}} \cdot \gamma_{Cl^-}} \quad (3)$$

Another example of solid $CaHPO_4$ formation is given by:

$$Ca^{2+} + HPO_4^{2-} \leftrightarrow CaHPO_4\ (s) \quad (4)$$

For solid precipitation, we have a solubility product:

$$K_{CaHPO_4} = a_{Ca^{2+}} \cdot a_{HPO_4}{}^{2-} = m_{Ca^{2+}} \cdot m_{HPO_4}{}^{2-} \cdot \gamma_{Ca^{2+}} \cdot \gamma_{HPO_4}{}^{2-} \quad (5)$$

where the activity of the solid is put to unity on the mole fraction scale and therefore does not appear in the equation. The $K_{CaCl}{}^+$ and K_{CaHPO4} used here are intrinsic constants. They pertain to activities and can apply only to concentrations at infinite dilution. For not very diluted solutions (ionic

strength > 0.01 mol/kg water), the values of activity coefficients determine the activities of ion species together with the concentration according to equation (1). The values of activity coefficient can be obtained by using models, or by experiment but then mean activity coefficients and activities must be used in the case of ions; due to the requirement of electro-neutrality, it is not possible to do measurements with separate ions. Various models have been developed to describe ion activity coefficient in solutions, such as the Debye-Hückel limiting law, extended Debye-Hückel equation, Guggenheim equation and Davies equation. The details of these models have been reviewed by van Boekel (2008) and will not be explained in this thesis. The Davies equation (Davies, 1962) is an empirical model that has been widely used because it gives reasonable prediction of the ion activity coefficient for a solution with an ionic strength up to 0.5 mol/kg water. The Davies equation is written as follows:

$$\log(\gamma_i) = -0.5\ z_i^2 \left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.2\ I\right),\ (I < 0.5\text{mol/kg water}) \quad (6)$$

where z_i is the charge of ion species i, I represents the effective ionic strength, which can be calculated as follows:

$$I = \frac{1}{2}\sum_i m_i \cdot z_i^2 \quad (7)$$

However, all models are approximations in describing ion activity coefficients. The accuracy of model calculation highly depends on the complexity of the system. For a multicomponent system, ion pairs/complexes formation must be taken into account for the calculation of ionic strength. Otherwise, errors may exist for the values of activity coefficients.

1.3 Ion equilibria – Effects on milk proteins

Aggregation and gelation of denatured dairy proteins can be induced by the presence of divalent cations (Bryant & McClements, 2000). The proposed mechanism is divalent cation bridging, in which two negatively charged regions on the protein are brought together by electrostatic attraction to the positive ion. Together with hydrophobic interaction, divalent cation bridging via colloidal calcium phosphate is thought to stabilize the casein micelle (Horne, 1998). During acidification, colloidal calcium phosphate is solubilized (Dalgleish & Law, 1989), and the kappa-casein micellar tails are collapsing (Vasbinder et al., 2003). The subsequent aggregation of casein has been proposed to be driven by a complex interaction including hydrophobic, van der Waals, and calcium-bridging attractions (Lucey, 2002).

Some direct evidence for the important role of salts in milk systems can be found in recent literature. The increase in ionic strength by adding NaCl, has a significant effect on ion speciation in milk such as a decrease in pH, an increase in Ca^{2+} concentration, a decrease in the activity coefficient of ionic species and an increase in the hydration of casein micelles (Gaucheron, 2005; Huppertz & Fox, 2006). As a result, several properties of milk are changed, e.g., the rennet coagulation time of milk is increased (Famelart et al., 1999; Van Hooydonk et al., 1986) and the ethanol stability is reduced (Huppertz & Fox, 2006). Puvanenthiran et al. (2002) showed a difference in gel hardness in whey-supplemented acid gels with differing lactose and mineral compositions. The authors also found that in the case of the highest mineral concentration, no gelation was observed at all. Philippe et al. (2003) studied the physicochemical properties of skim milk enriched with $CaCl_2$. The results showed that the behaviour of colloidal calcium phosphate seemed to be unchanged whatever the added calcium concentration. However, addition of

Ca to skim milk caused an increase in micellar density and resulted in a more compact micellar core. Choi et al. (2007) demonstrated that the concentration of colloidal calcium phosphate (CCP) associated with the casein micelles had an important influence on the properties of rennet-induced gels. Udabage et al. (2001) reported that addition of citrate or EDTA reduced the storage modulus of rennet-induced gels and above certain concentration rennet gelation was inhibited completely (10 mmol/kg milk). Mizuno and Lucey (2007) investigated the properties of milk protein gelation formed by addition of tetrasodium pyrophosphate (TSPP). The authors proposed a mechanism in which TSPP-induced gelation occurs when the added TSPP acts with calcium as a cross-linking agent between dispersed caseins. This provided fundamental information on the interactions between emulsifying salts and caseins that could be useful for the control of textural and functional properties of processed cheese. In summary, it is clear that control of the specific ion equilibria in dairy systems provides a way to control the functional properties of milk proteins and their gels.

1.4 Ion equilibria – Quantification

In general, the total ion concentrations in milk can be determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) for cations and by anion exchange chromatography for anions (Asfaw & Wibetoe, 2005; Gaucheron et al., 1996; Nobrega et al., 1997). However, the concentrations and activities of free divalent cations, such as Ca^{2+} and Mg^{2+}, are known to play more important roles than the total ion concentrations in the physicochemical properties of casein micelles. Ca^{2+} and Mg^{2+}, along with pH, affect the stability of the caseinate system and its behaviour during milk processing, especially in the coagulation of milk by rennet, heat and ethanol (Fox & McSweeney, 1998). The free Ca^{2+} ions can influence the environment

of the negatively charged casein micelles, leading to enhancement or reduction of their stability (Horne & Parker, 1981). Traditional methods for measuring Ca^{2+} activity or concentration in milk are based on the cation exchange resin technique (Christianson et al., 1954; Holt et al., 1981; Muldoon & Liska, 1969; Van Kreveld & Van Minnen, 1955) and the murexide method (Holt et al., 1981; Tessier & Rose, 1958). However, these methods have disadvantages such as being time-consuming (Muldoon & Liska, 1969) and inaccurate (Holt et al., 1981; Neville et al., 1995). So far, the calcium ion selective electrode (Ca-ISE) (Geerts et al., 1983; Holt et al., 1981; Lin et al., 2006; Silanikove et al., 2003; Tsioulpas et al., 2007) has been used to rapidly determine free Ca^{2+} activity in milk. However, the Ca-ISE may suffer from matrix effects (Holt et al., 1981; Neville et al., 1995), which means that the composition of the sample may affect the accuracy of Ca^{2+} ion activity measurement. This requires that the calcium electrode must be properly calibrated in standards with composition similar to the samples, but such standards have not been developed yet. To measure Mg^{2+} activity or concentration, a cation exchange resin technique has been developed by Van Kreveld and Van Minnen (1955). However, this method has the same disadvantages as described for the measurement of Ca^{2+}. More recently, a new method named Donnan Membrane Technique (DMT), based on Donnan equilibrium in a negatively charged membrane, has been developed by Temminghoff et al. (2000) and used to measure concentrations of free metal ions simultaneously in soil water. The DMT has been shown to be a robust and accurate technique to simultaneously determine different metal ions. It seems worthwhile to explore the potential of DMT for determination of free metal ions in food system.

1.5 Ion equilibria – Ion speciation models

Ion speciation models that describe the ion equilibria of milk-like systems have been developed since the 1980s. Wood et al. (1981) attempted to calculate the ion concentrations in a simulated milk salt solution. For the ease of calculation, assumptions were made in the model such as no presence of magnesium, phosphate esters and proteins, and potassium was regarded as equivalent to sodium. Lyster (1981) extended the model from Wood et al. (1981), by including an equilibrated solid phase at different temperatures and pH values. Holt et al. (1981) developed a model to describe the ionic composition in milk diffusate, including all the relevant components in milk diffusate except milk proteins, and the calculated free Ca^{2+} and Mg^{2+} concentrations were in reasonable agreement with experiment. These models have been used for many years as useful tools to describe the ionic composition in aqueous phase of milk or dairy products (Gaucheron et al., 2000; Kent et al., 1998; Le Graët & Gaucheron, 1999; Morris et al., 1988; Philippe et al., 2003), and to understand the interrelationship between the major ions and the physicochemical properties of casein micelles. However, these models did not include the colloidal calcium phosphate (CCP) and casein-bound cations in casein micelles. Therefore, their application in prediction of ion partitioning in milk and dairy products is still limited. More recently, Holt (2004) developed an equilibrium thermodynamic model for calculation of the ion partitioning in milk in which the colloidal calcium phosphate is assumed to be in the form of calcium phosphate nanoclusters. A generalized empirical formula for the calcium phosphate nanoclusters was used to define the molar ratios of small ions (Ca, Mg, P_i and citrate) to a casein phosphorylated sequence (Holt, 2004). This model provided a reasonable prediction of ion partitioning in milk in comparison with the experimental results of White and Davies (1958, 1963). Still, this model

requires further validation with more ion compositional data of milk samples under different conditions. Mekmene et al. (2009) presented a model to predict the ion partitioning between the micellar phase and the aqueous phase in milk and mineral-enriched milks (e.g., with added NaCl, $CaCl_2$, Na_3Citrate and Na_2HPO_4). This latter model is based on the earlier model of Holt et al. (1981) by integrating interactions of cations in casein micelles and taking into account the solubility of calcium phosphate in the micellar phase. The model was validated to give predictions generally agreeing with the literature experimental results, though differences in the order of 10 to 20 % still remain between model and experimental results. Moreover, this model is used to predict ion partitioning in milk and milk enriched with different electrolytes at a fixed pH value of 6.75. However, in reality, addition of electrolytes to milk induces changes in pH. Very recently, Mekmene et al. (2010) presented a model to simulate the ion equilibria of milk upon acidification. The model gave reasonable predictions of ion equilibria compared to experimental results. However, their model is not a generic model to describe the ion equilibria of milk at various conditions. Moreover, magnesium is considered to exist in the micellar phase as $Mg_3citrate_2$, which is an incorrect assumption since Mg is not present in the form of $Mg_3citrate_2$ as reported by Alexander and Ford (1957). Consequently, a generalized model to describe the ion equilibria of milk under various conditions should be developed.

1.6 Aim and outline of this thesis

The objective of the research described in this thesis is first to quantify ion equilibria in milk and milk-derived systems as affected by practical conditions (e.g. with addition of different electrolytes and non-electrolytes). Second, we aim to develop ion speciation models that can be used to predict

the behaviour of ions in milk and milk derived systems as a function of changing conditions.

After this introductory chapter, **Chapter 2** describes the application of the Donnan Membrane Technique (DMT) in milk-like systems. DMT was investigated for its potential to determine the concentrations of free Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} ions in milk-like systems.

Simulated milk ultrafiltrate solution (SMUF) solution represents the milk serum in terms of mineral composition. **Chapter 3 and Chapter 4** focus on developing models for ion equilibria of SMUF as a start for further establishment of ion equilibria model for milk.

Earlier studies showed that sugars, as non-electrolyte, have significant effects on Ca^{2+} ion activities and pH in buffer solutions, but without offering reasonable explanations. **Chapter 5** studies the influence of various disaccharides on ion equilibria in milk-like systems by using a more recent theory named Mean Spherical Approximation (MSA).

Based on the models for ion equilibria of SMUF, a model named Milk Ion Speciation (MIS) was built and tested for milk under various conditions, as shown in **Chapter 6**.

Ca-ISE was reported to suffer from matrix effects. It is investigated whether the ion compositions of calibration standards have an effect on the ion activity coefficients and liquid junction potential, thereby affecting the Ca^{2+} activity. **Chapter 7** focuses on developing new calibration standards for Ca-ISE to improve the accuracy of Ca^{2+} activity measurements.

Chapter 8 is the general discussion in which the developed ion speciation models are critically discussed in terms of limitations and opportunities. Moreover, the practical use of the models is tested for a concentrated system derived from one-month-old Cheddar cheese, to investigate the predictive capacity of the models developed.

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Chapter 2

Simultaneous determination of free Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} ion concentrations in simulated milk ultrafiltrate and reconstituted skim milk using the Donnan Membrane Technique

This Chapter has been published as:
Gao, R., Temminghoff, E. J. M., Van Leeuwen, H. P., Van Valenberg, H. J. F., Eisner, M. D., & Van Boekel, M. A. J. S. (2009). Simultaneous determination of free calcium, magnesium, sodium and potassium ion concentrations in simulated milk ultrafiltrate and reconstituted skim milk using the Donnan Membrane Technique. *International Dairy Journal, 19*, 431-436

Abstract

This study focused on determination of free Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} concentrations in a series of $CaCl_2$ solutions, simulated milk ultrafiltrate (SMUF) and reconstituted skim milk using a recently developed Donnan membrane technique (DMT). A calcium ion selective electrode was used to compare the DMT results. The study showed that the free Ca^{2+} concentrations measured by the DMT agreed well with calcium electrode data. Concentrations of free Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} measured by the DMT agreed with concentrations predicted by existing ion speciation models. It is concluded that the DMT is suitable to measure various free metal ion concentrations simultaneously in complex milk-type systems.

Keywords: free Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} concentration, Donnan Membrane Technique (DMT), calcium ion selective electrode, simulated milk ultrafiltrate (SMUF), reconstituted skim milk

2.1 Introduction

The mineral fraction is a small fraction of milk (approximately 8-9 g L^{-1}), and contributes to the nutritional quality of milk and largely determines the physicochemical state of milk, thereby determining the conformation and stability of milk proteins (Fox & McSweeney, 1998; Gaucheron, 2005; Walstra & Jenness, 1984).

Potassium, sodium, calcium and magnesium ions are the main cations in milk. The monovalent cations Na^+ and K^+ are present mainly in the free form and only to a limited extent in the form of ion pairs. The divalent cations, Ca^{2+} and Mg^{2+}, play roles in the physicochemical properties of casein micelles, such as gelation induced by acid and rennet, heat stability, ethanol stability and sediment formation (Fox & McSweeney, 1998; Lin et al., 2006).The free Ca^{2+} ions can influence the environment of the negatively charged casein micelles, leading to enhancement or reduction of the repelling forces among them (Horne & Parker, 1981). Calcium contributes to the integrity of casein micelles by forming linkages between the protein molecules either as colloidal calcium phosphate (CCP) or by directly binding to caseins (Holt, 1992; Schmidt, 1982). Therefore, it is important to be able to accurately measure the activity and the concentration of free calcium and magnesium in milk.

Traditional methods for measuring Ca^{2+} activity or concentration are based on the cation exchange resin technique (Christianson et al., 1954; Holt et al., 1981; Muldoon & Liska, 1969; Van Kreveld & Van Minnen, 1955) and the murexide method (Holt et al., 1981; Tessier & Rose, 1958). The cation exchange resin technique is quite time-consuming (Muldoon & Liska, 1969)

and there is competition between Ca^{2+} and Mg^{2+} (Temminghoff et al., 2000), which could hinder accurate determination of Ca^{2+} concentration. Furthermore, Holt et al. (1981) indicated that this method may overestimate Ca^{2+} concentration if citrate or inorganic phosphate complexes of metals are bound by the resin. The murexide method gave higher values for free Ca^{2+} concentration in milk when compared to the resin method and a calcium ion selective electrode (Holt et al., 1981). This is possibly due to the alterations of the calcium complex formation by the murexide (Neville et al., 1995). Since the 1980s, the calcium ion selective electrode (Geerts et al., 1983; Holt et al., 1981; Lin et al., 2006; Silanikove et al., 2003; Tsioulpas et al., 2007) has been used to rapidly determine ionic calcium activity in milk. The electrode may suffer from a matrix effect (Holt et al., 1981; Neville et al., 1995), and some systems may be more sensitive to matrix effects than others (Neville et al., 1995). Consequently, the calcium electrode must be properly calibrated to determine the free calcium activity and concentration. To measure Mg^{2+} activity or concentration, a cation exchange resin technique has been developed by Van Kreveld and Van Minnen (1955). However, this method has the same disadvantages as described above.

Recently, a new method named Donnan Membrane Technique (DMT), based on the Donnan equilibrium, has been developed by Temminghoff et al. (2000) and used in the field of environmental technology to measure free metal ion concentrations. Oste et al. (2002) applied the DMT to measure zinc and cadmium binding by humic acid in a range of pH 4 to 8, and the DMT agreed well with the ion selective electrode measurement. Van der Stelt et al. (2005) successfully used modified DMT cells to determine Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} in animal slurry, which is a mixture of faeces, urine and cleaning water with high ionic strength (~ 0.4 M). Kalis et al. (2007) also successfully determined

the concentrations of free metal ions including copper, cadmium, zinc, nickel and lead in multi-component solutions in which natural organic matter complexes metal ions. Thus the DMT has been shown to be a robust and accurate technique to simultaneously determine different metal ions in multi-component solutions, with minimal distortion of sample equilibrium and interferences from other components.

The objective of this paper is to determine whether the DMT is applicable to measure the free calcium, magnesium, sodium and potassium concentrations in simulated milk ultrafiltrate (SMUF) and skim milk. Results will be compared with literature data (Holt et al., 1981; Lyster, 1981) and for Ca^{2+} with a calcium ion selective electrode method.

2.2 Materials and methods

2.2.1 Donnan Membrane Technique

The theory of the Donnan membrane technique was described by Temminghoff et al. (2000). Briefly, the sample solution, called the donor solution, containing free metal ions and their bound forms, is separated from an acceptor solution by a semi-permeable and highly negatively-charged cation exchange membrane. The negative electric potential of the membrane allows cations to pass through and restricts anions from crossing the membrane. The more positive the charge of an ion, the faster is equilibration between donor and acceptor side of the DMT cell. When the Donnan equilibrium is reached, the free cation (M^{z+}_{donor}) concentration in the donor is equal to the free cation ($M^{z+}_{acceptor}$) concentration in the acceptor at the same ionic strength on both sides. When the ionic strength is different between donor and acceptor, correction can be made based on the Donnan equilibrium

principle (Helfferich, 1962). This implies that, at the Donnan equilibrium, the activity ratios (corrected for charge) of the ions in the solutions on the two sides of the membrane are equal (Equation (1)):

$$\left\{\frac{\left(M^{z+}\right)_{donor}}{\left(M^{z+}\right)_{acceptor}}\right\}^{1/z} = \frac{\left(N^{+}\right)_{donor}}{\left(N^{+}\right)_{acceptor}} \quad (1)$$

(M^{z+}) means the activity of the free target cation with charge z in either acceptor or donor. (N^{+}) represents the activity of a monovalent reference ion (normally potassium ion K^{+}).

Potassium is chosen to correct for the differences in ionic strength between donor and acceptor. It exists almost completely as free ions (Van Der Stelt et al., 2005).

The changing metal ion concentration in the acceptor solution during equilibration can be described by the following equation (Equation (2)) (Kalis et al., 2007; Temminghoff et al., 2000)

$$[Me]_{acc,t} = [Me]_{acc,t=0} + ([Me]_{acc,t=\infty} - [Me]_{acc,t=0}) \times \{1 - \exp(-bt)\} \quad (2a)$$

$$t_{0.95} = \frac{\ln(20)}{b} \quad (\text{when } [Me]_{acc,\,t=0} \approx 0) \quad (2b)$$

where:

$[Me]_{acc,t}$ is the metal concentration in the acceptor solution at time t, $[Me]_{acc,\,t=0}$ is the metal concentration in the acceptor solution at time 0, $[Me]_{acc,\,t=\infty}$ is the metal concentration in the acceptor solution at the Donnan equilibrium, and b is a constant related to the ion transport dynamics.

The value $t_{0.95}$ is the time needed for the acceptor solution to reach 95% of

the Donnan membrane equilibrium. The $t_{0.95}$ is often used as a measure for the Donnan membrane equilibrium because full equilibrium is never reached in practice.

2.2.2 DMT apparatus

For this research, the field DMT cell, developed by Van der Stelt et al. (2005), to determine the free cation concentrations was used. **Figure 1** shows the DMT system set-up. The DMT cell consists of one chamber, which contains the acceptor solution. The acceptor chamber is separated from the sample (the donor) by two negatively charged cation exchange membranes (BDH Laboratory Supplies, Poole, UK). Each membrane is a matrix of polystyrene and divinylbenzene with sulphonic acid groups, which fully deprotonate above pH 2. The thickness of the membrane is 0.16 mm, and the charge density of the membrane is 0.8 mmol g^{-1} (Weng et al., 2005). The acceptor compartment, which contains an electrolyte solution with ionic strength similar to that of the donor, has a volume of ~10 mL and the contact surface area for cation exchange is ~20 cm^2. The large ratio of surface area to volume leads to fast cation exchange between sample (donor) and acceptor solution (Kalis et al., 2007). The acceptor chamber is completely immersed in the sample solution, which is continuously stirred to minimize diffusion gradients in the donor solution (Kalis et al., 2007). Samples are taken out of the donor and acceptor solution at regular intervals for the determination of cation concentrations. Before using the DMT cell, all the materials, except the membrane, were cleaned by successively washing with: 0.1 M HNO_3 and deionized water (Millipore, Amsterdam, The Netherlands). For the membrane, the cleaning procedure described by Kalis et al. (2007) was used.

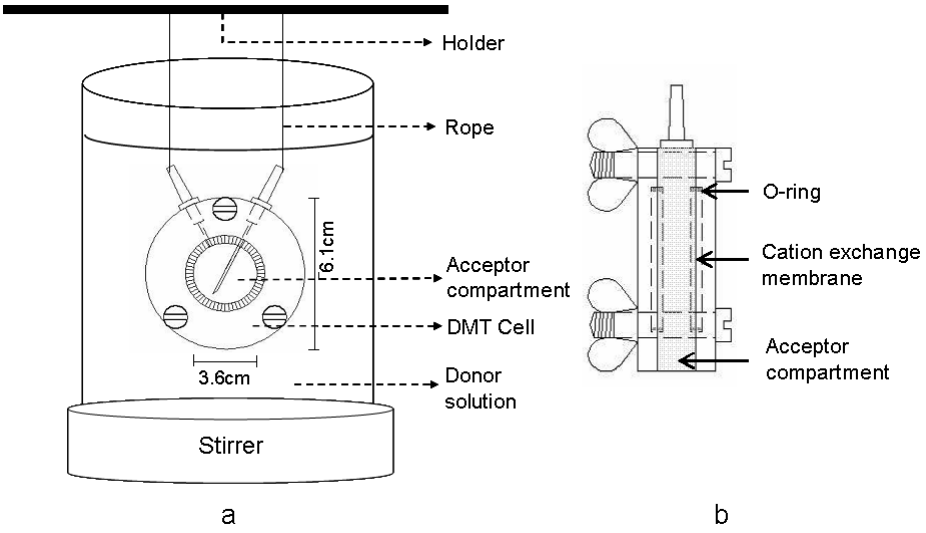


Figure 1 (a) Schematic presentation of the DMT set-up and front view of a field DMT cell. (b) Side view of a field DMT-cell.

2.2.3 Experimental Setup

To test the performance of the DMT to determine free metal ions, three systems of increasing complexity were employed: (i) a series of $CaCl_2$-KCl solutions, (ii) simulated milk ultrafiltrate (SMUF) and (iii) reconstituted skim milk. Experiments were carried out at 20 ºC and all glassware was washed in phosphate-free detergent, soaked in 1.4 M nitric acid and rinsed in deionized water (Millipore, Amsterdam, The Netherlands).

2.2.3.1 Ca^{2+} concentration determination in the $CaCl_2$-KCl solutions

The $CaCl_2$-KCl solutions used to test the DMT for ionized calcium concentration determination were: (1) 10 mM $CaCl_2$ and 20 mM KCl; (2) 5 mM $CaCl_2$ and 35 mM KCl; (3) 1 mM $CaCl_2$ and 47 mM KCl with an ionic strength of ~50 mM for each solution and final pH of each donor solution was 5.5. The acceptor solution (pH 5.5) contained 2 mM $Ca(NO_3)_2$ and 44

mM KNO_3 and had an ionic strength of approximately 50 mM. The pH was measured by a combined glass electrode (Orion 8172BNWP, Thermo, Beverly, USA). The DMT cell was filled with 10 mL acceptor solution and immersed into a beaker containing 2 L sample solution. The donor solution had been continuously stirred for 48 hours. Samples were taken out of the donor and the acceptor at the times 0, 4, 8, 24 and 48 h. Dilution was carried out if necessary. Calcium was determined by Inductively Coupled Plasma-Atomic emission Spectrometry (ICP-AES) (Varian, Mulgrave, Australia). Additionally, a calcium electrode (Orion 97-20, Thermo, Beverly, USA) was used as well to compare with the DMT results. The DMT experiment was carried out in duplicate, and the calcium electrode measurement repeated six times.

2.2.3.2 Ca^{2+}, Mg^{2+}, K^+ and Na^+ determination in simulated milk ultrafiltrate (SMUF)

A lactose-free simulated milk ultrafiltrate (SMUF) was prepared according to Jenness & Koops (1962). The acceptor solution consisted of 2 mM $Ca(NO_3)_2$ and 60 mM KNO_3, which had an ionic strength similar to SMUF. The pH of SMUF was 6.60. The DMT cell was filled with 10 mL acceptor solution and was immersed in a beaker containing 2 L SMUF solution. A same procedure was used for sampling and measuring as described in section 2.2.3.1. The experiment was repeated six times.

2.2.3.3 Ca^{2+}, Mg^{2+}, K^+ and Na^+ determination in reconstituted skim milk

Low heat skim milk powder (Nilac, NIZO, the Netherlands) was reconstituted by dissolving 200 g milk powder in 1800 g deionized water (Millipore, Amsterdam, The Netherlands) at 40-45 ºC under gentle stirring

for 45 minutes. Sodium azide 0.04 % (w/w) was added to prevent bacterial growth. The reconstituted skim milk was stored at 20 ºC overnight. The acceptor solution consisted of 5 mM $Ca(NO_3)_2$ and 65 mM KNO_3, having an ionic strength of ~80 mM, which is similar to that of milk. The DMT cell was immersed into 2 L skim milk, and the skim milk was continuously stirred for 72 hours. Samples were taken out of the donor and the acceptor solutions at 0, 4, 8, 24, 48 and 72 h. Skim milk samples were first ultracentrifuged (at 100,000 g, 20 ºC for 1 hour) in a Beckman L-60 ultracentrifuge with a 70 Ti rotor (Beckman Instruments Inc, California, USA). The supernatants were collected and diluted for the total concentration analysis by ICP-AES (Varian, Mulgrave, Australia). The samples of the acceptor were diluted and measured by ICP-AES (Varian, Mulgrave, Australia). A calcium electrode (Orion 97-20, Thermo, Beverly, USA) was used to measure the calcium activity in both the supernatant and the reconstituted skim milk, with the purpose first to compare with the DMT results and second to determine if casein micelles interfere with the determination of Ca^{2+} concentration by the electrode as reported by Silanikove et al. (2003). Ca^{2+} ion concentrations in supernatants were recalculated to Ca^{2+} ion concentrations in skim milk by multiplying by a correcting factor 0.96, in order to account for the excluded volume by proteins. The experiment was repeated four times.

2.2.3.4 Ionic calcium measurement by a calcium electrode

Ionic calcium was determined using an Orion 97-20 ion-plus calcium electrode and an Orion 720A+ meter (Thermo, Beverly, USA). Calibration was carried out with freshly prepared standard solutions in the range between 10^{-4} and 10^{-2} M $CaCl_2$. Calibration standard solutions were adjusted to ionic strength 0.09 M with KCl to calibrate the electrode for measurement of all the samples. The calibration curve plotted the electric potential (mV) as a

function of logarithm of calculated calcium activity. The time necessary for a stable reading was approximately 5 min. The obtained calcium activity for each sample was converted into free calcium concentration using the Davies equation (Davies, 1962) accounting for the calcium ion activity coefficient, as shown in the following formula:

$$\mathrm{Log}(\gamma\ \mathrm{Ca}^{2+}) = -0.5\ (z\ \mathrm{Ca}^{2+})^2\ (\frac{\sqrt{I}}{1+\sqrt{I}}\ - 0.2\ I) \qquad (3)$$

in which $\gamma\mathrm{Ca}^{2+}$ is the activity coefficient of free Ca^{2+}, $z\mathrm{Ca}^{2+}$ is the charge number of free Ca^{2+}, and I is the effective ionic strength of the sample, expressed in M unit.

The free Ca^{2+} concentration is then calculated by the following equation:

$$c\mathrm{Ca}^{2+} = \frac{a\mathrm{Ca}^{2+}}{\gamma\mathrm{Ca}^{2+}} \qquad (4)$$

in which $a\mathrm{Ca}^{2+}$ is the free Ca^{2+} activity, and $c\mathrm{Ca}^{2+}$ is the free Ca^{2+} concentration, expressed in M unit.

2.2.3.5 Chloride and phosphate determination

Less positively charged inorganic ion pairs and complexes, such as CaCl^{+}, MgCl^{+} and $\mathrm{CaH_2PO_4}^{+}$, may pass through the membrane. Therefore, total chloride and phosphate were also monitored in the acceptor solution to determine whether significant amount of chloride and phosphate were present at equilibrium. The chloride and phosphate were measured over time in the acceptor solution using FIAstar 5000 Analyzer (FOSS Tecator AB, Höganäs, Sweden) and ICP-AES (Varian, Mulgrave, Australia), respectively. Samples were removed from the acceptor solutions at 0, 8, 24 and 48 h in both the $\mathrm{CaCl_2}$-KCl experiment and the SMUF experiment; at 0, 8, 24, 48 and 72 h in the skim milk experiment. The total phosphorus was expressed as $\mathrm{PO_4}$,

because no organic phosphate was present in the acceptor.

2.2.3.6 Statistical analysis

Statistical analysis of the experimental data was carried out. The t-test was applied to test the significance of differences between the results obtained by the calcium electrode method and the DMT method at a level of $P < 0.05$

2.3 Results and discussion

2.3.1 Free Ca^{2+} concentration in a series of $CaCl_2$-KCl solutions

The DMT was tested first for Ca^{2+} determination with a series of $CaCl_2$-KCl solutions. **Figure 2a** illustrates the attainment of Donnan equilibrium over time for the ratio of $Ca_{acceptor}$ / Ca_{donor}. After 8 hours, more than 95 % Donnan equilibration was achieved for each sample. The ionic calcium transports were well described by equation (2a) with *b* values estimated for the fit to the data of calcium ion. No significant differences in pH were found between donor and acceptor solutions during the equilibration. At 8h, the concentration of chloride in the acceptor solution was less than 1 % of the concentration in the donor solution, indicating that a very limited amount of chloride ions was transported over the membrane and hardly affecting the free Ca^{2+} concentration, as shown in **Figure 2a**.

The free calcium concentrations determined by DMT are shown in **Table 1** together with the ion selective electrode (ISE) data. The DMT result is consistent with the electrode result for the 10 mM $CaCl_2$ sample. For samples with 5 mM and 1 mM $CaCl_2$, the DMT results differ slightly from the electrode results but there is no statistically significant difference in the results between the two methods ($P < 0.05$). This validates DMT in its

measuring Ca^{2+} concentrations in simple salt solutions.

Table 1 Free calcium ion concentration determined by DMT and calcium electrode for a series of $CaCl_2$-KCl solutions [a].

Solutions	DMT (mM)	Electrode (mM)
$CaCl_2$ 10 mM	8.9 ± 0.6	8.7 ± 0.4
$CaCl_2$ 5 mM	4.5 ± 0.2	4.4 ± 0.2
$CaCl_2$ 1 mM	0.91 ± 0.06	0.87 ± 0.04

[a] Measurements using the DMT and the calcium selective electrode were repeated two times and six times, respectively; ± means standard deviation; ionic strength of each $CaCl_2$ solution was approximately 0.05 M.

2.3.2 Ca^{2+}, Mg^{2+}, K^+ and Na^+ concentrations in simulated milk ultrafiltrate (SMUF)

It took about 6 hours for Ca^{2+} and Mg^{2+} to reach the Donnan equilibrium whereas for Na^+ 24 hours were required (**Fig 2b**). The transport of ions can be described well by the empirical equation (2a) with *b* values estimated for the fit to the data of the individual ions. At 24 h, the concentrations of chloride and phosphate in the acceptor were < 3% and < 1% of those in the donor (**Fig 2b**), respectively, which means that almost no metal phosphate complexes and metal chloride ion pairs were formed at Donnan equilibrium in the acceptor solution. In other words, the Ca^{2+}, Mg^{2+}, Na^+ and K^+ concentrations measured in the acceptor solution, represent the free Ca^{2+}, Mg^{2+}, Na^+ and K^+ concentrations at the Donnan equilibrium. During the equilibration, the pH and calcium activity of SMUF remained constant whereas the pH of the acceptor changed from 6.2 to 6.4. This slight change in pH did not affect cation concentration because it hardly changed the ionic strength of the acceptor and donor, and complexation with phosphate is then

insignificant.

Table 2 shows the cation concentrations determined by the DMT and the calcium electrode as well as values reported by Lyster (1981) in SMUF. Both the DMT and the calcium electrode gave repeatable results (standard deviation less than 10 % of the mean value). Moreover, the free Ca^{2+} concentrations determined by the DMT and the calcium electrode were in fair agreement with the values calculated by Lyster (1981). However, free Mg^{2+} concentrations showed a large difference between the DMT result and the calculated result. This difference may be due to the association constant of $MgCitrate^-$ complex used in Lyster's model, in which the pKa value of $MgCitrate^-$ was -3.29 (Lyster, 1981). However, Holt et al. (1981) used a pKa value for $MgCitrate^-$ of -5.05 (Martell & Smith, 1979). In this case, the free Mg^{2+} concentration would be reduced in the calculation, which is most likely comparable to the DMT result.

Table 2 Free Ca^{2+}, Mg^{2+}, Na^+ and K^+ concentrations determined by DMT and calcium electrode in SMUF [a] (n=6).

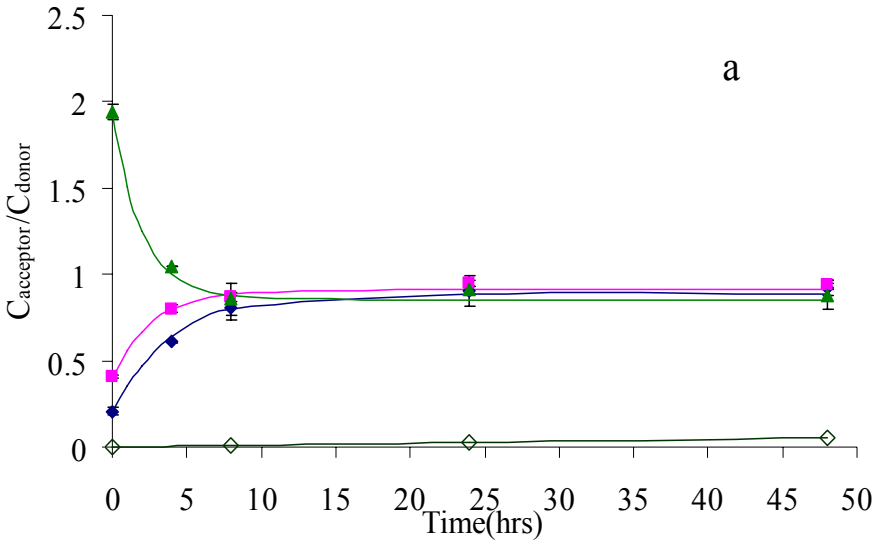
Ions	DMT (mM)	Electrode (mM)	Literature data (mM) [b]
Ca^{2+}	1.58 ± 0.03	1.38 ± 0.03	1.23
Mg^{2+}	0.53 ± 0.01	-	2
Na^+	17.0 ± 0.1	-	-
K^+	38.5 ± 0.3	-	-

[a] [Ca] = 9.17 ± 0.07 mM, [Mg] = 3.31 ± 0.03 mM, [Na] = 19.0 ± 0.1 mM, [K] = 40.5 ± 0.3 mM, [P_i] = 11.88 ± 0.16 mM, [Cl] = 38.2 ± 1.4 mM, [Citrate] = 9.6 mM, [SO_4] = 1 mM; ± means standard deviation; ionic strength of SMUF was approximately 0.067 M

[b] Values were taken from Lyster (1981) who calculated the free metal ion

concentrations in a simulated milk salt solution, which contained [Ca] = 9 mM, [Mg] = 3.2 mM, [Na] = 57.7 mM, [P_i] = 11.6 mM, [Cl] = 37 mM, [Citrate] = 9.6 mM.

Additionally, the Ca^{2+} concentration determined by the calcium electrode (1.38 ± 0.03 mM) was lower than that obtained by the DMT (1.58 ± 0.03 mM). This difference could be due to the error in predicting free ion activity coefficients by the Davies equation (see section 2.2.3.4), which does not take into account certain ion pair formation. Indeed, not only 1:1 ion pairs but also highly complexed ion pairs are formed in SMUF, thereby affecting the actual ionic strength and the free ion activity coefficients. Also, the calcium electrode is possibly affected by a matrix effect. Holt et al. (1981) reported that Ca^{2+} concentration determined by different methods followed the order ion selective electrode < calculated ≈ resin equilibrium < murexide. The DMT showed Ca^{2+} concentration consistent with Lyster's and Holt's results, which suggested that the DMT results are sound.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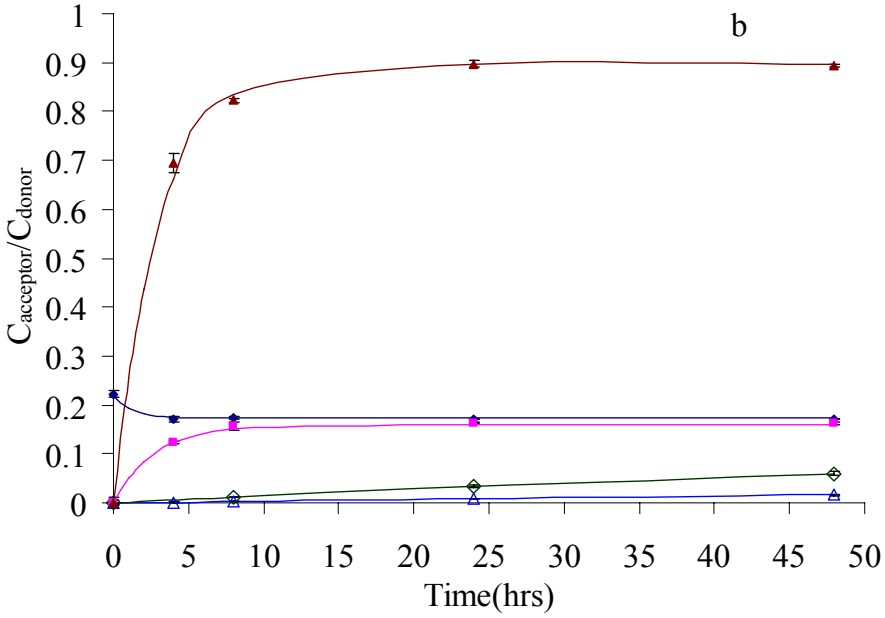
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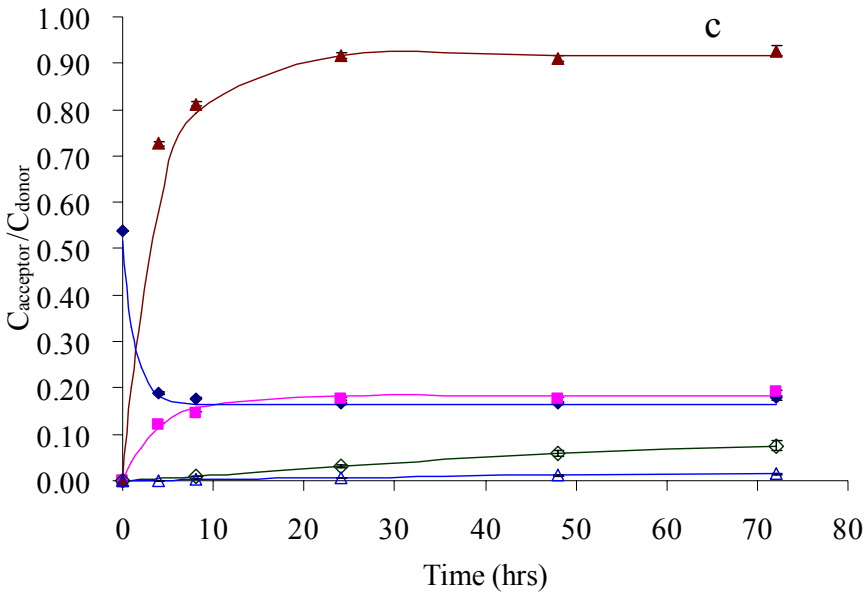

Figure 2 (a) Acceptor/donor concentration ratio for Ca^{2+}: (▲) 1mM $CaCl_2$; (■) 5mM $CaCl_2$ (◆) 10mM $CaCl_2$ and Cl^- (◇) as a function of time. (b) Acceptor/donor concentration ratio in SMUF for Ca^{2+} (◆), Mg^{2+} (■), Na^+(▲), Cl^- (◇) and phosphate (△) as a function of time. (c) Acceptor/donor concentration ratio in reconstituted skim milk for Ca^{2+} (◆), Mg^{2+} (■), Na^+(▲), Cl^- (◇) and phosphate (△) as a function of time. Curves are based on modeled values using equation (2a).

2.3.3 Ca^{2+}, Mg^{2+}, K^+ and Na^+ concentrations in reconstituted skim milk

To test a more complex system, experiments were conducted to measure the cations in reconstituted skim milk. **Figure 2c** shows that Donnan equilibrium

was reached after 8 hours for Ca^{2+} and Mg^{2+} and after 24 hours for Na^{+}. During the experiments, the reconstituted skim milk had constant pH and calcium activity, indicating that the original ion equilibrium of milk was not altered. The pH of the acceptor changed from 6.0 to 6.2. Some chloride and phosphate passed through the membrane with time. However, chloride and phosphate had no effect on free cation concentrations in the acceptor due to their low concentrations which were less than 3 % of the total chloride and 1 % of the total phosphate in the donor, respectively, as shown in **Figure 2c**.

Table 3 shows the cation concentrations determined by the DMT and the calcium electrode and calculated by Holt et al. (1981). The calcium and magnesium ions were mostly complexed by anions in the serum, whereas the potassium and sodium ions were largely free. Both the DMT and calcium electrode gave repeatable and comparable values for Ca^{2+} concentrations, which are within the typical range 1-3 mM (Demott, 1968; Holt, 1985). The concentrations of Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} which were determined by DMT, were generally consistent with the calculated ions concentrations in milk diffusate by Holt et al. (1981). Differences seems to be due to total Ca, Mg Na and K ion concentrations in Holt's calculation a bit higher than those determined in DMT. Moreover, no statistically significant difference was found between the Ca^{2+} concentration determined by the calcium electrode and the DMT ($P < 0.05$).

The Ca^{2+} activity, determined by calcium electrode in milk serum obtained by ultracentrifugation, was the same as that in skim milk, implying that the calcium electrode was not disturbed by complexing substances, such as casein micelles. This is in disagreement with the results of Silanikove et al. (2003), who reported that casein micelles in skim milk interfered with the

determination of Ca^{2+} concentration with an calcium electrode. From our findings as shown in Table 3, we conclude that it is not necessary to exclude casein micelles in measurement of Ca^{2+} activity and concentration in skim milk at neutral pH.

Table 3 Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} concentrations determined by DMT and calcium electrode in the reconstituted skim milk [a] (n=4).

Ions	DMT (mM)	Electrode [b] (mM)	Electrode [c] (mM)	Literature data [d]
Ca^{2+}	1.56 ± 0.06	1.58 ± 0.01	1.62 ± 0.02	2.00
Mg^{2+}	0.58 ± 0.02	-	-	0.81
Na^{+}	14.9 ± 0.2	-	-	20.9
K^{+}	40.8 ± 0.6	-	-	36.29

[a] Total ion concentrations in skim milk serum: [Ca] = 9.06 ± 0.05 mM, [Mg] = 3.15 ± 0.05 mM, [Na] = 17.3 ± 0.3 mM, [K] = 42.8 ± 0.7 mM, [P_i] = 11.3 ± 0.3 mM, [Cl] = 42.0 ± 0.4 mM, [Citrate] = 9.4 ± 0.2 mM.

± means standard deviation; ionic strength of skim milk was approximately 0.08 M

[b] Measurement of Ca^{2+} concentration in the serum of the reconstituted skim milk

[c] Measurement of Ca^{2+} concentration in the reconstituted skim milk

[d] Calculated concentrations of free metal ions in a typical milk diffusate (Holt et al., 1981), in which total ion concentrations were [Ca] = 10.2 mM, [Mg] = 3.4 mM, [Na] = 22.0 mM, [K] = 38.0 mM, [P_i] = 12.4 mM, [Cl] = 32.3 mM, [Citrate] = 9.4 mM.

2.4 Conclusion

The Donnan membrane technique (DMT) is demonstrated to be able to simultaneously analyze various free metal ion concentrations in SMUF and reconstituted skim milk, without any interference from the matrix and without disturbing the original ionic equilibria of the samples. The method provides reliable results, which are generally in good agreement with the calcium electrode results and literature data. The DMT is promising and has large potential applications in dairy research. The DMT offers possibilities for measuring the free ion concentrations, such as, Mg^{2+}, Zn^{2+} in milk for which no electrodes are available. Moreover, it may measure the free Ca^{2+} and Mg^{2+} concentration in milk at different temperatures, as indicated by Sata (2004) that the cation exchange membrane is stable up to 120 °C. This is an advantage over the calcium electrode, which can not be used at pasteurization temperature. DMT has the disadvantage of being slow. However, it has been recently developed to measure free ion concentration in dynamic systems, in which the equilibration time was largely reduced (30 minutes instead of 2 days) (Marang et al., 2006). Thus, the method can be possibly used to measure Ca^{2+} concentration in milk during acidification. Lastly, the DMT is expected to measure free metal ion concentrations in viscous dairy products, e.g. yoghurt and pudding. Therefore, the Donnan membrane technique can be helpful in exploring the ionic equilibria in milk quantitatively understanding more physicochemical interactions between casein micelles and ionic species.

Acknowledgements

We thank Johan Uijtenbroek and Peter Nobels, from the Department of Soil Quality, Wageningen University, for performing part of the experimental

work.

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Chapter 3

Modelling ion composition in simulated milk ultrafiltrate (SMUF) I. Influence of calcium phosphate precipitation

This Chapter has been published as:
Gao, R., van Halsema, F. E. D., Temminghoff, E. J. M., van Leeuwen, H. P., van Valenberg, H. J. F., Eisner, M. D., Giesbers, M., & van Boekel, M. A. J. S. (2010). Modelling ion composition in simulated milk ultrafiltrate (SMUF) I. Influence of calcium phosphate precipitation. *Food Chemistry, 122*, 700-709

Abstract

Freshly prepared simulated milk ultrafiltrate (SMUF) is a solution that is supersaturated with respect to various calcium phosphate phases that precipitate in time. As a consequence, the ion composition of equilibrated SMUF was found to be significantly different from that of freshly prepared SMUF. This study proposes a thermodynamic ion-speciation model that is able to describe ion equilibria in SMUF. Moreover, it is also able to describe calcium phosphate precipitation in fresh SMUF on its way to equilibrium by using an apparent solubility product for $CaHPO_4 \cdot 2H_2O$ as a function of time. The model was validated by experiments in which $CaCl_2$ and Na_2HPO_4 were added to freshly prepared SMUF. The changes in calcium activity and pH were followed and the precipitates were characterized by X-ray diffraction. The model was able to predict the observed changes.

Keywords: calcium phosphate, precipitation, ion equilibria, ion speciation model, SMUF

3.1 Introduction

Ion equilibria of milk, and in particular the distribution of calcium phosphate between casein micelle and serum phase, play an essential role in the stability and structural integrity of casein micelles (Fox & McSweeney, 1998; Holt, 1997; Walstra & Jenness, 1984). Alterations of ion equilibria can have important effects on casein micelles and this can have major consequences for milk processing, such as coagulation caused by rennet, acid and heating (De La Fuente, 1998). Therefore, it is necessary to have an accurate description of the ion equilibria in terms of concentrations and activities of various ions and complexes. Milk minerals are partitioned between the colloidal and aqueous phase. Approximately 70% of calcium, 30% of magnesium, 50% of phosphate and 10% of citrate are present as undissolved complexes in casein micelles, named colloidal calcium phosphate (CCP) (Holt, 1997). In the milk serum phase, salts exist either as free ions or as ion pairs. Sodium and potassium ions form only weak ion pairs with chloride, citrate and phosphate and therefore mainly remain as free ions. The divalent ions (Ca^{2+} and Mg^{2+}) are mainly complexed with citrate and to a lesser extent with HPO_4^{2-}. There is a dynamic and very rapidly attained equilibrium between the ion pairs and the free ions. Also, there is a dynamic but slowly attained equilibrium between salts in the aqueous phase and colloidal phase (Walstra et al., 1984).

Simulated milk ultrafiltrate (SMUF), which was first described by Jenness and Koops (1962), is a solution that mimics the ion composition of milk ultrafiltrate. It is the most widely used simplified milk system in milk mineral deposition studies (Andritsos, Yiantsios & Karabelas, 2002; Rosmaninho & Melo, 2006; Spanos, Patis, Kanellopoulou, Andritsos & Koutsoukos, 2007).

It can also be used as a buffer solution for dispersion of milk proteins or for studying physico-chemical properties of milk proteins, such as heat stability, electrophoretic and ultracentrifugal analyses (Jenness et al., 1962). However, studies (Andritsos et al., 2002; Lyster, 1979; Schmidt & Both, 1987) have shown that SMUF is supersaturated with respect to various calcium phosphate phases when casein micelles are absent. This means that SMUF is thermodynamically unstable and subject to precipitation of calcium phosphate.

The main calcium phosphate phases involved are amorphous calcium phosphate $Ca_3(PO_4)_2$ (ACP), dicalcium phosphate dihydrate $CaHPO_4{\cdot}2H_2O$ (DCPD), octacalcium phosphate $Ca_4H(PO_4)_3{\cdot}2.5H_2O$ (OCP), tricalcium phosphate $Ca_3(PO_4)_2$ (TCP) and hydroxyl-apatite $Ca_5(PO_4)_3OH$ (HAP). HAP is the least soluble calcium phosphate phase. However, it may not be the one that precipitates first because its precipitation kinetics is slower than that of the other phosphate forms (Rosmaninho et al., 2006). The formation of calcium phosphate precipitates depends on environmental conditions such as pH, ionic strength, temperature, molar ratio of Ca/P, presence of other ions (Johnsson & Nancollas, 1992; Schmidt et al., 1987; Schmidt, Both, Visser, Slangen & Vanrooijen, 1987; Spanos et al., 2007; Van Kemenade & De Bruyn, 1987). Earlier studies (Johnsson et al., 1992; Mekmene, Quillard, Rouillon, Bouler, Piot & Gaucheron, 2009; Van Kemenade et al., 1987) have shown that pH is the key factor that determines the crystalline structure of calcium phosphates. DCPD and OCP are formed in more acidic conditions, whereas HAP is preferentially formed under neutral or basic conditions. In addition, other ions such as magnesium and citrate have been reported to act as inhibitors of HAP crystal growth (Johnsson et al., 1992; Rosmaninho et al., 2006). Instead, the presence of these ions in solution (Johnsson et al., 1992)

may stabilize the DCPD and OCP crystals. Consequently, calcium phosphate precipitation in SMUF is involved with several crystallization processes and transformation of various calcium phosphate phases and thus is quite complex.

Modelling studies of calcium phosphate precipitation in milk systems have rarely been carried out. Lyster (1981) developed an ion equilibria model for SMUF with the possibility to calculate calcium phosphate precipitation in equilibrium at different values of temperature and pH. However, this model only accounts for one solid phase at equilibrium, and it has not been validated to calculate ion equilibria in the presence of solid phases upon addition of salts to SMUF.

The objectives of the present work were twofold. The first objective was to develop an ion speciation model to calculate ion composition in SMUF at thermodynamic equilibrium, i.e., in the presence of precipitates. For this purpose, it was investigated how the model would perform by incorporating the solubility products of various calcium phosphate phases and other solids, such as calcium citrate, calcium carbonate and magnesium phosphate. In order to validate the model, experiments were carried out with SMUF and with SMUF to which $CaCl_2$ and Na_2HPO_4, were added, respectively, after which the system was allowed to reach equilibrium in the pH range between 4.8 and 7.4. The second objective was to develop an ion speciation model able to describe the ion composition of freshly prepared SMUF, i.e., far from equilibrium without any solid precipitation, and the change in ion composition while the system is equilibrating, i.e., under kinetic conditions of ongoing precipitation. For this purpose, a kinetic study was carried out to determine the change in the apparent solubility product of certain calcium

phosphate over time.

3.2 Materials and Methods

3.2.1 Experimental Setup

All experiments were carried out at 20 ± 1.0 ºC and all glassware was washed in phosphate-free detergent, soaked in 1.4 M nitric acid and rinsed in deionized water (Millipore, Amsterdam, The Netherlands). All chemicals used were purchased from Fluka, with 99.5 % purity. SMUF without lactose was prepared according to Jenness & Koops (1962).

3.2.1.1 Addition of $CaCl_2$

$CaCl_2$ was added to SMUF samples up to a concentration of 0.1 mol/kg water. For the experiments in which Ca^{2+} activity, pH and precipitates were measured and analysed, $CaCl_2$ solutions were added to SMUF to study the kinetics of precipitates formation. The equilibration time was approximately 8 or 9 days. Free Ca^{2+} and Mg^{2+} ion concentrations were determined by Donnan Membrane Technique (DMT) and the experimental procedure was described earlier by Gao et al. (2009). For the DMT experiment, solid $CaCl_2$ was added to SMUF to accelerate calcium phosphate precipitation. The observed acceleration was presumably due to a seeding action of the added crystals. It was checked that the Ca^{2+} activity and pH 24 h after adding solid $CaCl_2$ was the same as the values in SMUF 8 days after adding $CaCl_2$ solution. Also, X-ray diffraction showed no difference in the precipitates after adding $CaCl_2$ as solid or as solution. The DMT cell, which contains 10 mL acceptor solution, was immersed in 1.6 L donor solution (SMUF sample). The acceptor solution consisted of $Ca(NO_3)_2$ and KNO_3, and had ionic strength similar to the donors. Specifically, for concentration of added $CaCl_2$

in SMUF up to 0.04 mol/kg, the acceptor solution consisted of 5 mmol/kg $Ca(NO_3)_2$ and 113 mmol/kg KNO_3; for concentration of added $CaCl_2$ ranging from 0.06 and 0.1 mol/kg, the acceptor solution consisted of 10 mmol/kg $Ca(NO_3)_2$ and 247 mmol/kg KNO_3.The donor was continuously stirred for 48 hours. Samples were taken out of the donor and acceptor after 24 and 48 hour. Dilution was carried out if necessary. Calcium was determined by Inductively Coupled Plasma-Atomic emission Spectrometry (ICP-AES) (Varian Vista-Pro radial system, Mulgrave, Australia). The DMT experiment was carried out in duplicate, and the Ca activity and pH measurements were repeated six times.

3.2.1.2 Addition of Na_2HPO_4

Disodium phosphate (Na_2HPO_4) solution was added to SMUF in the concentration range between 0 and 0.48 % (w/w). Similarly to 3.2.1.1, the calcium activity and pH were measured during the equilibration period, and each measurement was repeated six times.

3.2.1.3 Precipitate analysis

SMUF with added salts ($CaCl_2$ or Na_2HPO_4) showed precipitation. Several steps were carried out to collect the precipitate for analysis. The first step was to allow the precipitates in SMUF to reach equilibrium, which was supposed to have happened when the pH and Ca activity did not change in time anymore. Second step was to centrifuge SMUF solution at 3000 rpm for 15 minutes at 20 °C (ALC PK131R, The Netherlands). After discarding the supernatant, precipitate was dried in an oven at 100 °C for 8 hours. The dried precipitate was acidified with 1.4 N HNO_3 and the corresponding acidified solution was diluted 1000 times. Elements like Ca, Mg, Na, K and Phosphor were measured by ICP-AES (Varian Vista-Pro radial system, Mulgrave,

Australia). The molar ratios of Ca/P and Mg/P were calculated to determine which precipitates were formed. In addition, the obtained precipitates were further identified by means of X-ray diffraction (XRD), as described in section 3.2.6.

3.2.2 Description of the model

The computer program AESolve (Halotec Instruments, The Netherlands) was used in order to calculate the ion composition of SMUF under different conditions. Two types of models were developed: one based on intrinsic thermodynamic constants describing equilibrium and the other describing the temporal changes of freshly prepared SMUF on its way to equilibrium. The first one was called EIS (Equilibrium Ion Speciation) model, and the second one was named DIS (Dynamic Ion Speciation) model.

The following components were included in the model: calcium, magnesium, sodium, potassium, citrate, phosphate, sulphate, chloride, and carbonate. Lactose and proteins were left out since the work presented here is limited to lactose-free SMUF. The components are supposed to react with each other, resulting in the formation of free ions, ion complexes, and salt precipitates. Ion complexes between major cations and major anions, as shown in **Table 1**, were considered in the model (Holt, Dalgleish & Jenness, 1981), while solid precipitation, such as dicalcium phosphate dihydrate (DCPD), and gas-liquid equilibria, such as partition of CO_2 between gas and aqueous phase, were included as well. Some examples of ion complexes and precipitates are:

$$Ca^{2+} + Citrate^{3-} \leftrightarrow CaCitrate^{-}\,(aq) \quad (1)$$

$$Ca^{2+} + HPO_4^{2-} \leftrightarrow CaHPO_4\,(aq) \quad (2)$$

$$Ca^{2+} + Cl^{-} \leftrightarrow CaCl^{+}\quad (aq) \quad (3)$$

$$Ca^{2+} + HPO_4^{2-} + 2H_2O \leftrightarrow CaHPO_4.2H_2O\,(s) \quad (4)$$

$$3Ca^{2+} + 2Citrate^{3-} + 4H_2O \leftrightarrow Ca_3Citrate_2.4H_2O\ (s) \quad (5)$$

The mass balance equation for each component was constructed and consisted of a sum of the concentrations of the various forms. As an example, the total concentration of calcium is shown in equation (6). The concentration of each ion species, for instance $CaCitrate^-$, can be calculated using equation (7), while the activity coefficients were calculated from the Davies equation (8).

$$\begin{aligned}[Ca]_{Total} = {} & [Ca^{2+}] + [CaCitrate^-] + [CaHCitrate] + [CaH_2Citrate^+] + [CaPO_4^-] \\ & + [CaHPO_4](aq) + [CaH_2PO_4^+] + [CaCl^+] + [CaHCO_3^+] + [CaOH^+] + [CaSO_4](aq) \\ & + 3\cdot[Ca_3Citrate_2.4H_2O](s) + [CaHPO_4.2H_2O](s) + 3\cdot[Ca_3(PO_4)_2](s) + [Ca(OH)_2](s) \\ & + [CaCO_3](s)\end{aligned} \quad (6)$$

$$[CaCitrate^-] = K_{CaCitrate^-} \times [Ca^{2+}] \times [Citrate^{3-}] \times \frac{\gamma_{Ca^{2+}} \times \gamma_{Citrate^{3-}}}{\gamma_{CaCitrate^-}} \quad (7)$$

$$Log(\gamma_{Ca^{2+}}) = -0.5(z_{Ca^{2+}})^2 \times \left(\frac{\sqrt{I_e}}{1+\sqrt{I_e}} - 0.2I_e\right) \quad (8)$$

in which $K_{CaCitrate^-}$ is the intrinsic association constant of $CaCitrate^-$ (see Equation (1)); $\gamma_{Ca^{2+}}$, $\gamma_{Citrate^{3-}}$, $\gamma_{CaCitrate^-}$ the molal activity coefficients of free Ca^{2+}, $Citrate^{3-}$ and $CaCitrate^-$, respectively; $z_{Ca^{2+}}$ is the charge number of free Ca^{2+}; I_e is the effective ionic strength of the sample, expressed in mol/kg water.

Intrinsic ion association constants (K_{ass}) and solubility products (K_{sp}) were taken from literature as shown in **Tables 1 and 2**. The K_{ass} and K_{sp} values indicated as estimated were not randomly chosen. The estimated values were derived on the basis of the agreement between model calculation and

experimental results in terms of pH, Ca^{2+} activity and concentration. K_{ass} and K_{sp} values of ion species and compounds related to sodium hexametaphosphate (SHMP) were also included in EIS and DIS models and were shown in the accompanying paper (part II) (Gao et al., 2009)

For solid precipitates, apparent solubility products of amorphous calcium phosphates were used in the DIS model for predicting clear SMUF solution or precipitation in SMUF with addition of certain salts at initial state. When dealing with precipitation at thermodynamic equilibrium state, solubility products such as crystalline $CaHPO_4.2H_2O$ and $Ca_3(PO_4)_2$ were employed in the EIS model.

The resulting equations describing electrolyte equilibria (called ion-speciation) are nonlinear algebraic equations that were solved numerically to obtain activities and concentrations for all the components and their complexes. Other properties of the solutions, such as the ionic strength, activity coefficients, pH and water activity, are also calculated by AESolve.

Table 1 Logarithm of intrinsic association constants (pK_{ass}) of ion species used in the EIS and DIS models

	H^+	Ca^{2+}	Mg^{2+}	K^+	Na^+
$Citrate^{3-}$	6.4[a]	5.22[a]	5.15[a]	1.53[e]	1.9[e]
$HCitrate^{2-}$	4.76[a]	3.3[c]	2.7[a]	1.6[e]	1.6[e]
$H_2Citrate^-$	3.13[a]	1.45[a]	1.18[a]	0[a]	0[a]
PO_4^{3-}	12.67[b]	6.46[a]	4.92[a]	1.7[e]	2[a]
HPO_4^{2-}	7.22[b]	2.85[a]	2.92[a]	1.11[a]	1.23[a]
$H_2PO_4^-$	2.17[b]	1.04[a]	1.08[a]	0[a]	0[a]
Cl^-	-	0.7[e]	1[e]	0[a]	0[a]
OH^-	-	1.3[b]	-	0[e]	0[e]
HCO_3^-	6.35[a]	1.26[b]	1.28[a]	0[a]	0[a]
SO_4^{2-}	-	2.31[a]	2.23[a]	0.85[a]	0.7[a]
$P_2O_7^{4-}$	9.25[b]	6.8[b]	7.2[b]	2.3[b]	2.3[b]
$HP_2O_7^{3-}$	6.6[b]	3.6[b]	3.06[b]	1.3[b]	1.3[b]
$H_2P_2O_7^{2-}$	2.4[b]	2.8[e]	2.5[e]	1[b]	1[b]
$H_3P_2O_7^-$	0.9[b]	2.3[b]	-	-	-
$H_2P_3O_{10}^{3-}$	2.5[b]	-	-	-	-
$HP_3O_{10}^{4-}$	6.54[b]	5.5[e]	6[e]	1.5[e]	1.7[e]
$P_3O_{10}^{5-}$	9.36[b]	8.1[b]	8.6[b]	2.5[b]	2.7[b]

[a] Holt et al. (1981)

[b] Smith and Martell (1981)

[c] Martell and Smith (1979)

[d] Davies (1962)

[e] Estimated

Table 2 Solubility products (K_{sp}) of typical solid precipitates employed in the EIS and DIS models.

Chemical name	Chemical formula	Solubility Product K_{sp}
Tricalcium citrate tetrahydrate	$Ca_3Citrate_2 \cdot 4H_2O$	$2.29 \cdot 10^{-18}$ [a]
Tricalcium phosphate (TCP)	$Ca_3(PO_4)_2$	$1.26 \cdot 10^{-29}$ [b]
Dicalcium phosphate dihydrate (DCPD)	$CaHPO_4 \cdot 2H_2O$	$1.87 \cdot 10^{-7}$ [c]
Amorphous tricalcium phosphate (ACP)	$Ca_3(PO_4)_2 \cdot xH_2O$	10^{-26} [d]
Amorphous dicalcium phosphate	$CaHPO_4 \cdot 2H_2O$	$1.66 \cdot 10^{-6}$ [e]
Octacalcium phosphate (OCP)	$Ca_4(HPO_4)_3 \cdot 5H_2O$	$1.26 \cdot 10^{-49}$ [c]
Calcium pyrophosphate	$Ca_2P_2O_7$	10^{-16} [e]
Magnesium pyrophosphate	$Mg_2P_2O_7$	10^{-16} [e]
Calcium carbonate	$CaCO_3$	$4.8 \cdot 10^{-9}$ [a]
Trimagnesium phosphate	$Mg_3(PO_4)_2$	10^{-24} [a]
Dicalcium sodium triphosphate	$Ca_2NaP_3O_{10}$	$6.31 \cdot 10^{-19}$ [e]
Tetracalcium hexametaphosphate	$Ca_4P_6O_{19}$	$3.16 \cdot 10^{-25}$ [e]

[a] Walstra & Jenness. (1984)

[b] Lyster (1981)

[c] Johnsson & Nancollas. (1992)

[d] Seely (1998)

[e] Estimated

3.2.3 Details of the computer program AESolve

The computer program AESolve (HaloteC Instruments, The Netherlands) allows users to perform complex equilibrium calculations in aqueous electrolyte systems. Solid precipitation and gas-liquid equilibria are taken

into account. AESolve generates a thermodynamically consistent mathematical model consisting of non-linear algebraic equations and their parameters for the selected components. The equations are solved numerically by the solver engine. Supported concentration units include mol/kg water (molality), g/kg water and mass percentage. Molarity (mol/l) is not used since it is temperature dependent. Furthermore, models that describe non-ideality of ions in the aqueous phase include the Debye-Hückel limiting law, extended Debye-Hückel and the Davies equation. The Davies equation was used in EIS and DIS models. AESolve can perform equilibrium calculation either for one point or for a range of points (for changing concentrations). Calculations can be chosen for fixed pH or water activity or for pH or water activity when the system has reached equilibrium.

Figure 1 shows a pseudo-algorithm to illustrate how AESolve works. An ion speciation model is defined by setting initial concentrations of selected components (in our case, the ones that were used to prepare SMUF). Subsequently, an iterative database search process starts that eventually yields all real species and involved reactions. If all species and involved reactions are known, the model is automatically built. The model consists of a set of algebraic equations and a set of unknown parameters, i.e., the concentrations to be calculated. The variables and expressions contained in the model are then parsed and compiled after which the order of evaluation is determined. In most cases large sets of algebraic loops need to be solved numerically. The total number of variables and equations may become large, since many real species will originate from only a small number of initial components through dissociation. AESolve automatically generates initial guesses with special algorithms. If a solution is not found after a specified time or number of iterations, the model is automatically simplified by

removing components or lowering concentrations in order to facilitate convergence. If a solution for this simplified model is found, the model is extended again. Previous results are then used as initial guesses for the larger model. This is also an iterative process until a solution is found for the entire model.

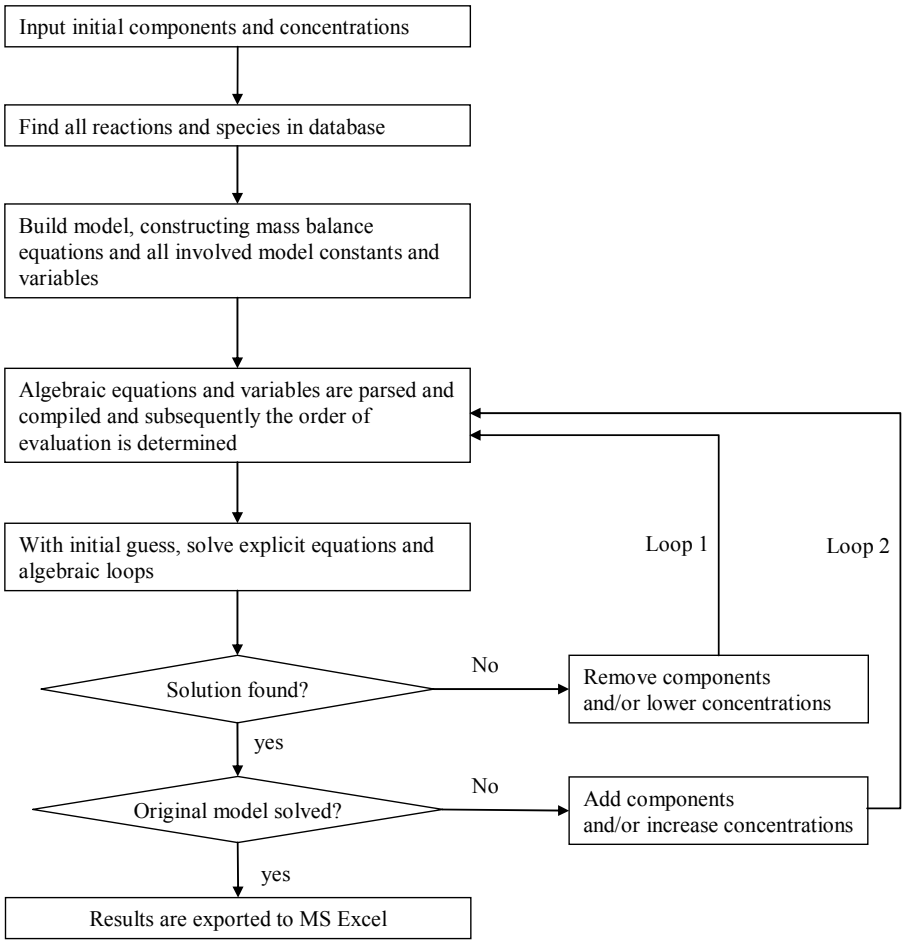

Figure 1 Schematic presentation of AESolve details

3.2.4 Donnan Membrane Technique

The theory of the Donnan membrane technique was described extensively by Gao et al. (2009) and Temminghoff et al. (2000). Briefly, the sample solution, called the donor solution is separated from an acceptor solution containing strong electrolytes by a semi-permeable and highly negatively-charged cation exchange membrane. The negative electric potential of the membrane allows cations to pass through and restricts anions from crossing the membrane. Within certain time, Donnan equilibrium is reached between the free metal ion (M^{z+}_{donor}) activity in donor and the free metal ion ($M^{z+}_{acceptor}$) activity in acceptor. The free metal ion activity present in the donor solution can be determined by correcting for differences in ionic strength between the donor and acceptor solution (Helfferich, 1962).

$$\left(\frac{aM^{z+}\,donor}{aM^{z+}\,acceptor} \right)^{1/z} = \frac{aN^{+}\,donor}{aN^{+}\,acceptor} \tag{9}$$

aM^{z+} represents the activity of the free target metal ion with charge z in either acceptor or donor. aN^{+} (normally potassium ion K^{+}) represents the activity of a monovalent reference ion. Potassium is chosen to correct for the differences in ionic strength between donor and acceptor (Van Der Stelt, Temminghoff & Van Riemsdijk, 2005).

If necessary, the so obtained free calcium activity in SMUF can be recalculated into free calcium concentration using the Davies equation (Davies, 1962) accounting for the calcium ion activity coefficient, as shown in equation (8).

The free calcium concentration is then calculated by the following equation:

$$m_{Ca^{2+}} = \frac{a_{Ca^{2+}}}{\gamma_{Ca^{2+}}} \times m^{\theta}_{Ca^{2+}} \tag{10}$$

in which a_{Ca}^{2+} is the free Ca^{2+} activity, and m_{Ca}^{2+} is the free Ca^{2+} concentration (mol/kg water), $m^{o}_{Ca}{}^{2+}$ is the standard state of free Ca^{2+} concentration (1 mol/kg water).

3.2.5 Ionic calcium determination by a calcium ion selective electrode

Ionic calcium activity was determined using an Orion 97-20 ion-plus calcium electrode and an Orion 720A+ meter (Thermo, Beverly, USA). Calibration was carried out with freshly prepared standard solutions in the range between 10^{-4} and 10^{-1} mol/kg water $CaCl_2$. All the standard solutions, except for 10^{-1} mol/kg water, were adjusted to ionic strength 0.09 mol/kg water with KCl. Calibration standard solutions were adjusted to ionic strength 0.09 mol/kg water with KCl to calibrate the electrode for measurement of all the samples. The calibration curve plotted the electric potential (mV) as a function of logarithm of calculated calcium activity. The time necessary to reach a stable reading was approximately 5 min, referring to electric potential change less than 0.1mV per minute.

3.2.6 X-Ray diffraction

X-ray diffraction (XRD) was conducted with a PANalytical Expert Pro System (Almelo, The Netherlands) by using nickel-filtered CuKα radiation (tube operating at 40 kV and 40 mA). The data were collected using an automated divergence slit (5mm irradiated length) and a 0.2 mm receiving slit. Phase identification was carried out with Crystallographica Search-Match software using the PDF data base release 2005 (McClune, 2005).

3.3 Results and Discussion

3.3.1 Precipitation at equilibrium: building of the model EIS

In order to build the model EIS, the following experiments were done. Salts were added to SMUF and the response was measured via Ca^{2+} and Mg^{2+} measurements, pH, and analyses of the precipitates formed.

3.3.1.1 Addition of $CaCl_2$ to SMUF

Addition of $CaCl_2$ to SMUF changed the original ion equilibria in terms of thermodynamic properties of ions, such as ion activity, ion concentration and ionic strength and resulted in precipitation. The Ca^{2+} activity at equilibrium increased nearly proportional to the amount of $CaCl_2$ added, as shown in **Figure 2a**. The Ca^{2+} activities measured by Ca-ISE were generally similar to the results by DMT, although there was a small difference at higher concentration of $CaCl_2$. The calculated Ca^{2+} activities were in general agreement with the experimental results. Besides Ca^{2+}, another major divalent cation Mg^{2+} was also influenced. **Figure 2b** shows that the free Mg^{2+} concentration at equilibrium initially increased and then slightly decreased as a function of the added $CaCl_2$ concentration. As an explanation for the observed behaviour, the EIS model suggests that extra Ca^{2+} associates with large quantity of citrate ions to form calcium citrate complex or precipitate, which then promotes dissociation of magnesium citrate complex, resulting in a sharp increase in Mg^{2+} concentration. With more and more $CaCl_2$, Mg^{2+} ion interacts with Cl^- to form the weak ion pair $MgCl^+$, leading to a slight decrease in the concentration of Mg^{2+} ions. The Mg^{2+} concentration predicted by the EIS model was in agreement with the experimental results. The ionic strength of SMUF was calculated to increase up to 0.291 mol/kg water

according to the EIS model, as illustrated in **Figure 2d**.

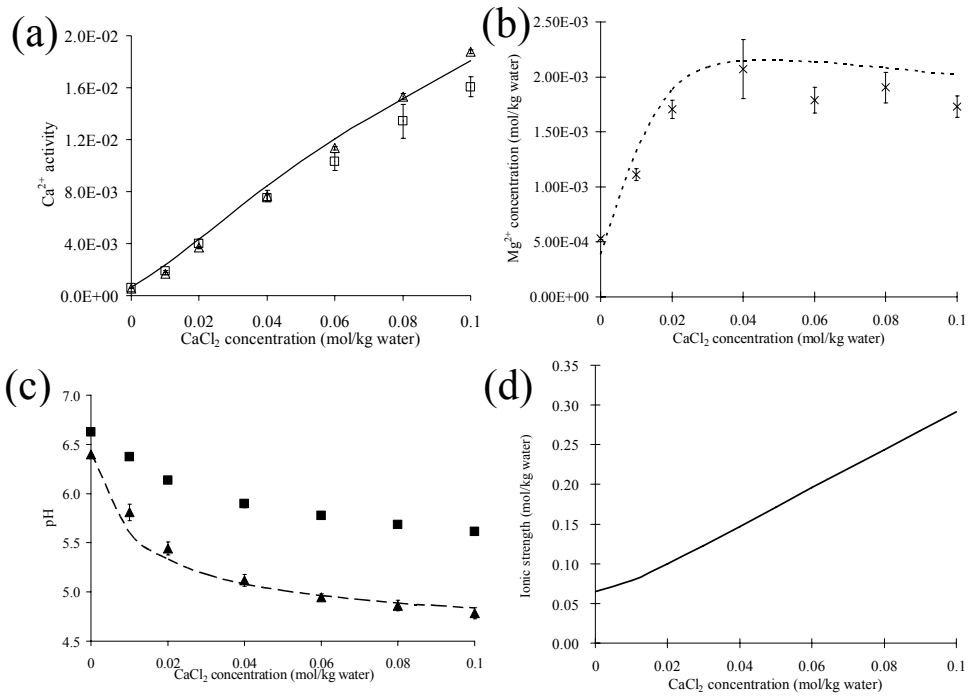

Figure 2 Effect of $CaCl_2$ addition on ion speciation in SMUF allowed to reach equilibrium. (a) Comparison of calculated and experimental free Ca^{2+} activity in SMUF: (□) DMT method (n=2); (△) Ca-ISE method (n=6); (—) EIS model calculation. (b) Comparison of calculated and experimental free Mg^{2+} concentration in SMUF: (×) DMT method (n=2); (---) EIS model calculation. (c) Comparison of calculated and experimental pH in SMUF: (■) measured pH at initial state (n=6); (▲) measured pH at equilibrium (n=6); (– –) EIS model calculation at equilibrium. (d) The calculated ionic strength.

As a result of adding $CaCl_2$, precipitation occurred in SMUF with respect to calcium phosphate. The EIS model showed that the precipitates were DCPD

($CaHPO_4.2H_2O$) and tricalcium citrate tetrahydrate ($Ca_3Citrate_2.4H_2O$) at equilibrium, which was confirmed by X-Ray diffraction as shown in **Figure 3A**. Due to the abundance of Ca^{2+} ions, SMUF became also supersaturated with tricalcium citrate, which therefore appeared to precipitate. In the pH range studied, DCPD was the single calcium phosphate phase formed. Below pH 6.7, DCPD and HAP are the most favourable calcium phosphate phases (Van Kemenade et al., 1987). However, HAP crystal formation is largely inhibited due to the presence of Mg^{2+} and Citrate. It is suggested that formation of DCPD was the main reason of a decrease of pH in SMUF upon addition of $CaCl_2$. The free Ca^{2+} ions interact with HPO_4^{2-} ions, resulting in deprotonation of $H_2PO_4^-$. Furthermore, adding $CaCl_2$ initially led to amorphous $CaHPO_4.2H_2O$ formation. With time, amorphous $CaHPO_4.2H_2O$ would be converted to its crystalline form. As a result, more precipitates were formed, causing further deprotonation of $H_2PO_4^-$ and a decrease in pH until equilibrium is reached. All these effects are reflected in the pH change shown in **Figure 2c**. Clearly, the pH at equilibrium was well predicted by the EIS model.

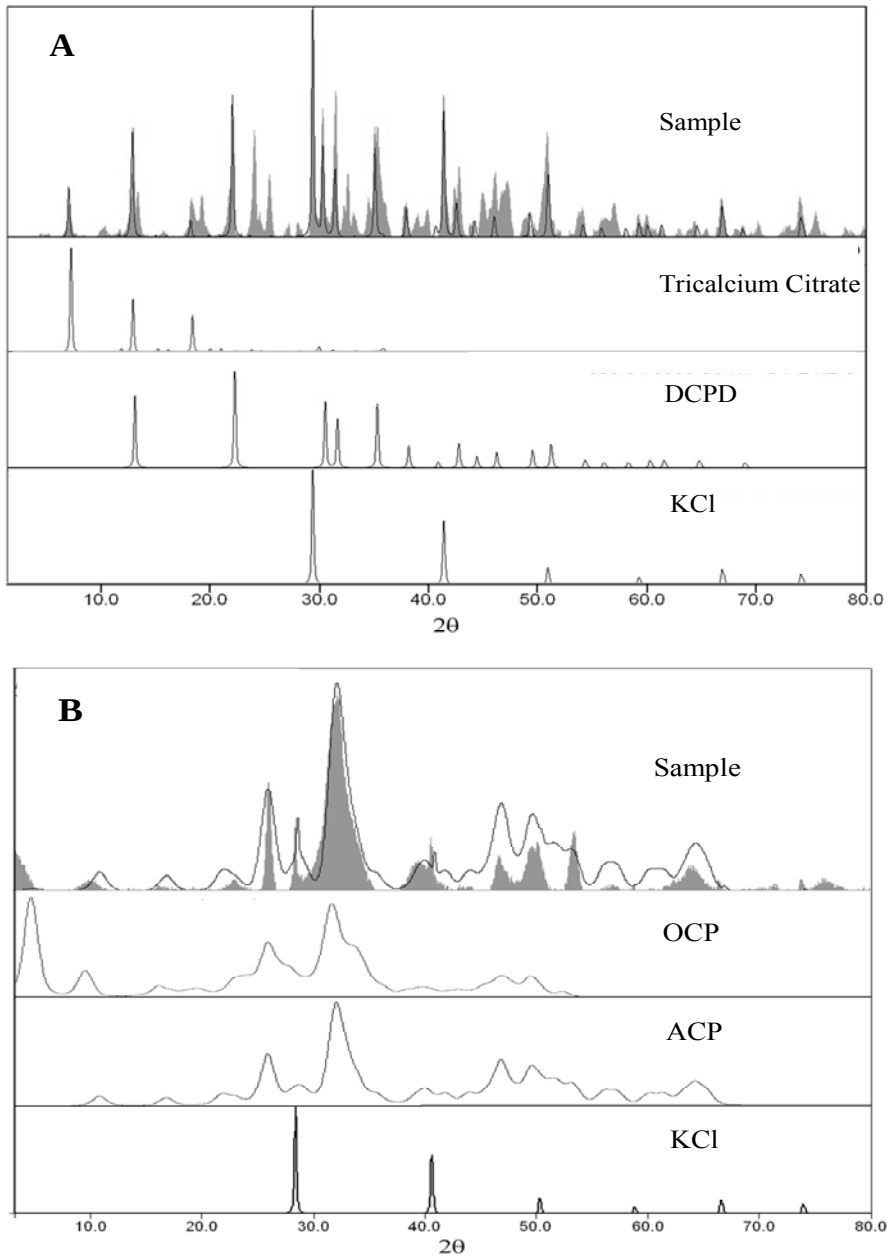

Figure 3 X-ray diffraction patterns of precipitates in SMUF: (A) $CaCl_2$ effect and (B) Na_2HPO_4 effect.

3.3.1.2 Addition of disodium phosphate (Na_2HPO_4)

Figures 4 (a) and **(b)** illustrate that after adding Na_2HPO_4, initially Ca^{2+} activities decreased and pH values increased as a function of Na_2HPO_4 concentration, respectively. With time, Ca^{2+} activities and pH values decreased until equilibrium. The Ca^{2+} activity and pH at equilibrium predicted by EIS matched with the experimental results within 10% deviation. The change of Ca^{2+} activity and pH in time is apparently related to the kinetics of calcium phosphate precipitation. **Figure 3B** shows a number of broad peaks indicating that the sample is quite amorphous. This suggested that the amorphous precipitates should be still in the process of calcium phosphate crystallization. The XRD pattern was best matched by $Ca_3(PO_4)_2$ (ACP) and $Ca_4H(PO_4)_3.5H_2O$ (OCP). Meanwhile, **Table 3** shows that the molar ratios of Ca/P are all between 1.33 and 1.45, indicating that the precipitates are most likely a mixture of OCP and ACP. Apart from the ACP and OCP peaks, some sharper peaks were also present in the pattern, which was crystalline KCl. Table 3 shows that the EIS model predicts OCP to be formed at equilibrium, no matter what concentration of Na_2HPO_4. According to precipitating sequence above pH 6.7 at 25 °C (Van Kemenade et al., 1987), which was DCPD/ACP → OCP → HAP, OCP was generally less soluble and more stable than DCPD and ACP. Therefore, it is unlikely that DCPD and ACP were present at equilibrium. Moreover, the presence of Mg^{2+} and citrate may play a role in preventing HAP crystal growth. It seemed that HAP was hardly formed. Therefore, the EIS model appeared to predict the behaviour of calcium phosphate precipitates at equilibrium.

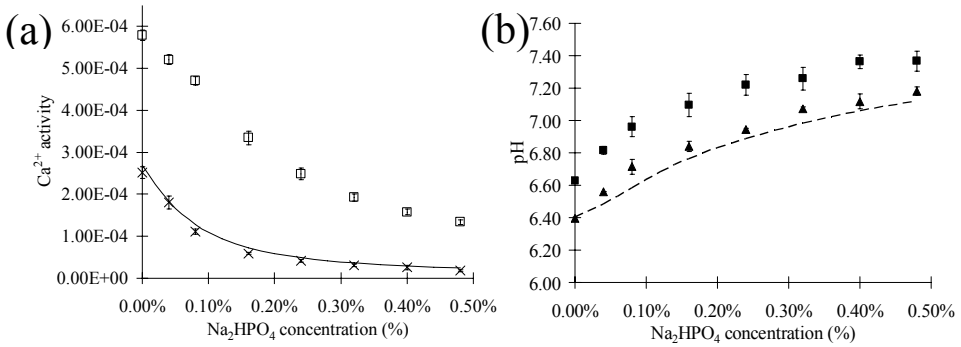

Figure 4 Effect of Na_2HPO_4 addition on ion equilibria in SMUF at equilibrium. (a) and (b) Comparison of calculated and experimental free Ca^{2+} activity and pH in SMUF, respectively: (□) and (■) represent free Ca^{2+} ion activity and pH, respectively at initial state (n=6); (×) and (▲) represent free Ca^{2+} ion activity and pH, respectively at equilibrium (—) and (– –) are free Ca^{2+} ion activity and pH predicted by the EIS model, respectively (n=6).

Table 3 Characteristics of calcium phosphate precipitates in SMUF with added Na_2HPO_4

Na_2HPO_4 (%) (w/w)	Experimental Ca/P	Calculated Ca/P	Precipitates by X-Ray	Precipitates by the EIS Model
0.04	1.45	1.33	$Ca_3(PO_4)_2$ & OCP	OCP
0.08	-	1.33	-	OCP
0.16	1.43	1.33	$Ca_3(PO_4)_2$ & OCP	OCP
0.24	-	1.33	-	
0.32	1.35	1.33	$Ca_3(PO_4)_2$ &OCP	OCP
0.40	1.36	1.33	$Ca_3(PO_4)_2$ & OCP	OCP
0.48	1.33	1.33	$Ca_3(PO_4)_2$ & OCP	OCP

3.3.1.3 Stability of SMUF

SMUF is a milk salt solution designed to have similar salt composition to milk serum. According to Jenness et al. (1962), freshly prepared SMUF is a clear solution. However, SMUF was observed to become turbid over time. **Figure 2c** and **Figure 4** show that the Ca^{2+} activity and pH of SMUF without addition of $CaCl_2$ or Na_2HPO_4 are reduced significantly. Furthermore, **Table 4** shows the Ca^{2+} activity is decreased over 50% and pH is decreased to 6.40. This can be explained by the formation of calcium phosphate precipitates. In other words, SMUF is a solution supersaturated with respect to calcium phosphate. Freshly prepared SMUF is therefore not in thermodynamic equilibrium and forms precipitates in time. The EIS model calculates the free Ca^{2+} activity and pH in SMUF at equilibrium, which is in good agreement with experimental results. Subsequently, **Table 5** shows the calculated ion equilibria of SMUF at equilibrium. It indicates that the precipitates mainly consist of DCPD and OCP with a molar ratio 99:1. According to solubility isotherms of calcium phosphate phases, in which DCPD was more stable below pH 6.7 at room temperature (Van Kemenade et al., 1987), it makes sense that DCPD was formed.

Table 4 Comparison of the measured calcium activity and pH (SD)[a] with the modelled results in freshly prepared SMUF and SMUF at equilibrium [b,]

	Ca^{2+} activity at t=0 h	Ca^{2+} activity at equilibrium	pH at t=0 h	pH at equilibrium
Measurement	5.4E-04 (1.5E-04)	2.70E-04 (2.0E-04)	6.63 (0.01)	6.40 (0.01)
Model	-	2.69E-04	-	6.40

[a] n=6

[b] Calcium activity was measured by calcium ion selective electrode

[c] modelled results were obtained according to EIS model

In summary, the EIS model was able to predict ion composition at equilibrium in SMUF and also in SMUF to which $CaCl_2$ or Na_2HPO_4 was added. The EIS model is strictly thermodynamic, i.e., based upon intrinsic thermodynamic association constants and solubility products. However, the consequence is that SMUF at equilibrium does not represent the salt composition in milk serum. This is of importance when SMUF is used as a buffer solution for dispersion of milk proteins or for studying the physico-chemical properties of milk proteins. Therefore, it was considered essential to also build a model that can predict ion composition in freshly prepared SMUF; we call this model DIS, as introduced above.

Table 5 Calculated concentrations (mmol/kg water) of ions and complexes in SMUF at equilibrium by EIS model [a, b].

		Cation complex			
Anion	Free ion	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
$Citrate^{3-}$	0.70	5.27	2.64	0.23	0.30
$HCitrate^{2-}$	0.23	0.05	-	0.14	0.08
$H_2Citrate^{-}$	- [c]	-	-	-	-
PO_4^{3-}	-	-	-	-	-
HPO_4^{2-}	1.77	0.14	0.09	0.34	0.25
$H_2PO_4^{-}$	5.98	0.02	0.01	0.14	0.08
Cl^{-}	32.3	0.04	0.05	0.74	0.41
OH^{-}	-	-	-	-	-
HCO_3^{-}	0.02	-	-	-	-
SO_4^{2-}	0.85	0.02	0.01	0.09	0.04
Free ion	-	0.65	0.39	36.5	20.2

[a] Concentrations (mmol/kg water) of major ions in freshly prepared SMUF: [Ca] = 9.0, [Mg] = 3.2, [Na] = 18.3, [K] = 39.2, [P_i] = 11.6, [Cl] = 33.5, [Citrate] = 9.6, [SO_4] = 1.0; ionic strength of SMUF was 0.069 mol/kg water

(calculated); pH of SMUF was 6.63.

[b] At equilibrium, calcium phosphate precipitated in SMUF, which were DCPD 2.70 mmol/kg water, and OCP 0.026 mmol/kg water. The ionic strength of SMUF was 0.065 mol/kg water (calculated) and pH of SMUF was 6.40.

[c] Concentration shown as: - < 0.01 mmol/kg water

3.3.2 Kinetic model of the change in pH and Ca^{2+} activity

In order to determine the ion composition in freshly prepared SMUF, the approach was to measure the Ca^{2+} activity or pH at the intermediate states between preparation and equilibrium. Next, the ion speciation model DIS was fitted to experimental data by adjusting the solubility product (K_{sp}) of DCPD. Subsequently, the obtained K_{sp} values were plotted against time and the corresponding curve was extrapolated to obtain the apparent K_{sp} at t=0.

SMUF to which $CaCl_2$ was added was chosen to obtain the apparent K_{sp} as a function of time, because in SMUF to which Na_2HPO_4 was added, transformation of various calcium phosphate phases took place, whereas this was not so with $CaCl_2$. There were two precipitates formed by adding $CaCl_2$ in SMUF. The formation of DCPD mainly accounted for the pH to change in time. Moreover, according to the precipitating sequence of calcium phosphate precipitates (Van Kemenade et al., 1987), DCPD appeared to be the first precipitating phase. Therefore, by adjusting the K_{sp} value of DCPD, the measured pH could be fitted by the DIS model at different times. By plotting the obtained K_{sp} values as a function of time, the apparent K_{sp} at t = 0 could be derived by extrapolation. However, for SMUF where Na_2HPO_4 was added, ACP was also formed initially. Due to the complexity of this system, the apparent K_{sp} of ACP at t=0 was not determined as described above. Several

solubility products of $Ca_3(PO_4)_2$ were found in literature, in which the difference between solubility products were up to 10^3. The highest K_{sp} of $Ca_3(PO_4)_2$ was chosen as the apparent K_{sp} of ACP at t = 0, which was 10^{-26} (Seely, 1998). Both the apparent K_{sp} of DCPD and ACP were employed in the DIS model to test whether the model gave good prediction in pH and Ca^{2+} activity at t = 0. It is stressed that this is a purely empirical way to tackle the kinetics of precipitation. It would require much more study on precipitation kinetics to find out the full details of the mechanism behind the observed phenomena.

Figure 5a shows the pH values at different times in SMUF with added $CaCl_2$. At intermediate states, the measured pH values were well fitted by the model by adjusting the K_{sp} values. The obtained K_{sp} values were plotted against time, as shown in **Figure 5b**, and were fitted to a polynomial equation. This equation was used to extrapolate to t = 0 and this resulted in an apparent K_{sp} = 5.78 ± 0.1 (95 % confidence interval). By using the apparent K_{sp} value at t = 0, the ion composition of the freshly prepared SMUF was calculated. With this value, no precipitates were predicted by the DIS model. The calculated pH and Ca^{2+} activity were 6.63 and 6.41E-04, respectively, which were comparable to the measured results as shown in **Table 4**. The measured Ca^{2+} activity was lower than the calculated result. This was probably because the calcium electrode was influenced by matrix effect (Gao et al., 2009). Instead, the Ca^{2+} activity measured by Donnan Membrane technique was 6.45E-04 (Gao et al., 2009), which was similar to the calculated value, indicating that the DIS model is indeed able to predict the ion composition at t = 0.

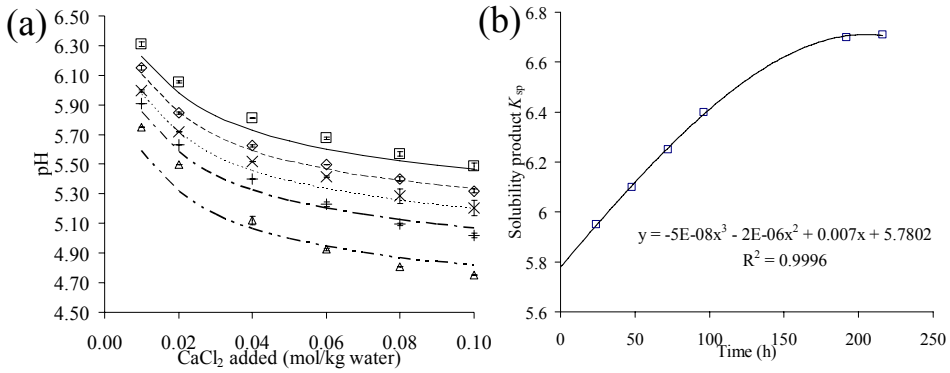

Figure 5 (a) model of pH change in time in SMUF with added $CaCl_2$. (□) measured pH at t = 24 h; (—) calculated pH at t = 24 h; (◇) measured pH at t = 48 h; (– –) calculated pH at t = 48 h; (×) measured pH at t = 72 h; (---) calculated pH at t = 72 h; (+) measured pH at t = 96 h; (– -) calculated pH at t = 96 h; (△) measured pH at t = 216 h; (– - -) calculated pH at t = 216 h; experiments were carried out in triplicate (b) mathematical calculation of solubility product of $CaHPO_4 \cdot 2H_2O$ at t =0.

The DIS model with apparent K_{sp} = 5.78 at t = 0 was also applied to calculate ion composition at t=0 in SMUF with added $CaCl_2$ and Na_2HPO_4. **Figure 6 (a)** shows that the model prediction of pH is in good agreement with the experimental results in freshly prepared SMUF with added $CaCl_2$. Likewise, the measured pH values matched well with the calculated results in SMUF with added Na_2HPO_4 at t = 0, as shown in **Figure 6b**. **Figure 6c** illustrates the calculated Ca^{2+} activities at t = 0; the model prediction was a bit higher but showed same tendency as the experimental results. The difference could be due to matrix effect interfering with calcium ion selective electrode.

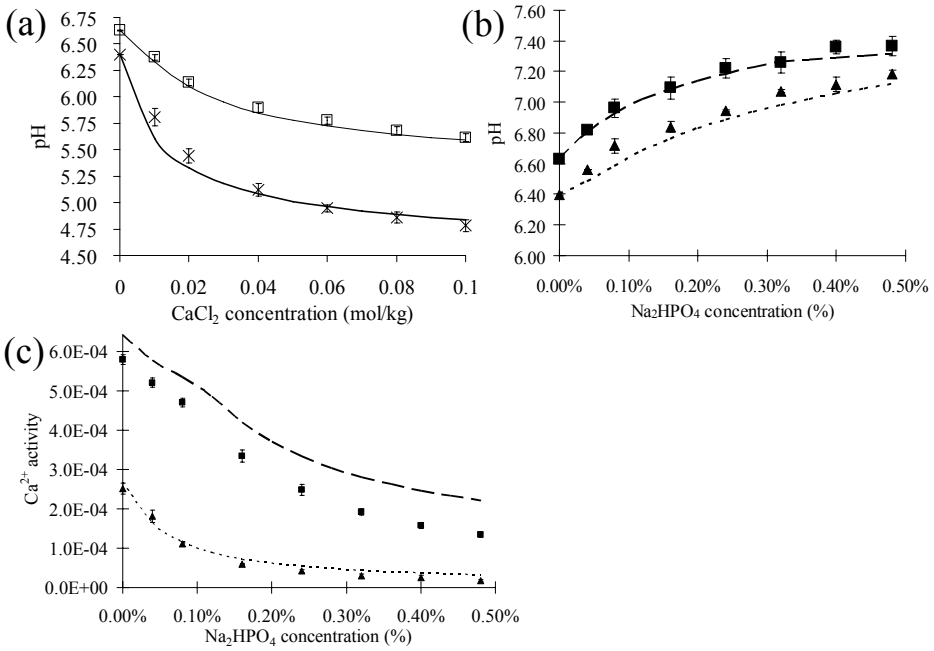

Figure 6 Comparison of calculated and measured pH and Ca^{2+} activity in SMUF. (a) $CaCl_2$ effect: (□) measured pH at initial state (—) calculated pH at initial state by DIS model; (×) measured pH at equilibrium (▬) calculated pH at equilibrium by EIS model. (b) and (c) Na_2HPO_4 effect on pH and Ca^{2+} activity, respectively: (■) measured pH or Ca^{2+} activity at initial state (– –) calculated pH or Ca^{2+} activity at initial state by DIS model; (▲) measured pH or Ca^{2+} activity at equilibrium (---) calculated pH or Ca^{2+} activity at equilibrium by EIS model.

3.3.3 Ion composition in freshly prepared SMUF

The ion species in SMUF, present either in a free form or complexed form, are involved in association or dissociation reactions. **Table 6** shows the calculated concentrations (DIS model) of ion species in SMUF at pH 6.63, which are generally in good agreement with literature data (Holt et al., 1981).

Summing up all the ion species, SMUF was calculated to have an ionic strength of 0.069 mol/kg water, which was similar to the reported value 0.073 mol/kg water (Holt et al., 1981). Divalent metal ions, e.g. Ca^{2+} and Mg^{2+}, were mainly bound by $citrate^{3-}$ and to a less extent by HPO_4^{2-}, while monovalent metal ions, e.g. Na^+ and K^+, were present mainly in the free form. The calculated (DIS model) concentrations of free Ca^{2+}, Mg^{2+}, Na^+ and K^+ ions matched well with reported experimental results (Gao et al., 2009), which were 1.58, 0.53, 17.0 and 38.5 mmol/kg water for the concentration of free Ca^{2+}, Mg^{2+}, Na^+ and K^+ ion, respectively. However, the calculated concentrations of each ion species, such as $CaCitrate^-$, and $NaCitrate^{2-}$, were slightly different from literature data (Holt et al., 1981). This could partly be due to the total concentrations of Ca, Mg, Na and phosphate from Holt et al. (1981) being a bit higher than those in SMUF, and partly because the association constants for certain ion species in the model were different from those by Holt et al. (1981).

Table 6 Calculated concentrations (mmol/kg water) of ions and complexes in freshly prepared SMUF by DIS model [a, b, c] versus literature values from Holt et al. (1981)

		Cation complex			
Anion	Free ion	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
$Citrate^{3-}$	0.38 (0.26)	6.61 (6.96)	2.20 (2.02)	0.12 (0.04)	0.16 (0.03)
$HCitrate^{2-}$	0.07 (0.04)	0.04 (0.01)	- (-)	0.04 (-)	0.02 (-)
$H_2Citrate^{-}$	- (-)	- (-)	- (-)	- (-)	- (-)
PO_4^{3-}	- (-)	- (-)	- (-)	- (-)	- (-)
HPO_4^{2-}	3.18 (2.65)	0.57 (0.59)	0.26 (0.34)	0.60 (0.52)	0.44 (0.39)
$H_2PO_4^{-}$	6.25 (7.50)	0.04 (0.07)	0.02 (0.04)	0.14 (0.18)	0.08 (0.10)
Cl^{-}	32.2 (30.90)	0.10 (0.26)	0.08 (0.07)	0.75 (0.68)	0.40 (0.39)
OH^{-}	- (-)	- (-)	- (-)	- (-)	- (-)
HCO_3^{-}	0.04 (0.32)	- (-)	- (-)	- (-)	- (-)
SO_4^{2-}	0.83 (0.96)	0.04 (0.07)	0.01 (0.03)	0.09 (0.10)	0.03 (0.04)
Free ion	-	1.59 (2.00)	0.62 (0.81)	37.5 (36.3)	17.2 (20.9)

[a] Concentrations (mmol/kg water) of the major ions in freshly prepared SMUF: [Ca] = 9.0, [Mg] = 3.2, [Na] = 18.3, [K] = 39.2, [P_i] = 11.6, [Cl] = 33.5, [Citrate] = 9.6, [SO_4] = 1.0; ionic strength of SMUF was 0.069 mol/kg water (calculated); pH of SMUF was 6.63.

[b] Values shown as ( ) were calculated concentrations (mmol/kg water) of ion species in a typical milk diffusate by Holt et al. (1981), in which [Ca] = 10.2, [Mg] = 3.4, [Na] = 22.0, [K] = 38.0, [P_i] = 12.4, [Cl] = 32.3, [Citrate] = 9.4, [SO_4] = 1.2; ionic strength was 0.073 M and pH was 6.70.

[c] Concentration shown as: - < 0.01 mmol/kg water

3.4 Conclusion

Freshly prepared SMUF is a solution that is thermodynamically unstable and

over time results in calcium phosphate precipitation. The thermodynamically based ion speciation model EIS appears to be able to successfully predict ion equilibria in the presence of calcium phosphate precipitate in equilibrated SMUF and SMUF to which $CaCl_2$ and Na_2HPO_4 are added. The DIS model on the other hand was found to be able to predict the ion composition of freshly prepared SMUF and that of SMUF on its way to equilibrium by incorporating the ongoing calcium phosphate precipitation by means of apparent solubility products. The DIS model was also able to calculate ion composition in freshly prepared SMUF to which $CaCl_2$ and Na_2HPO_4 were added. Also, the salt equilibria of freshly prepared SMUF by DIS are in good agreement with literature results. Nevertheless, the ion speciation model has some limitations. This model is only validated at room temperature. It does not yet take into account temperature effects on ion equilibria due to lack of availability of association constants and solubility products at various temperatures. In addition, the kinetics of calcium phosphate precipitation is, for the time being, only dealt with in an empirical way in the DIS model. To turn this into a mechanistic model, much more detailed research is required on crystallization and transformation of various forms of calcium phosphate. The current paper does not address the effects of other conditions (addition of HCl, ionic strength and polyphosphates), as it is subject to the accompanying paper (part II). An important conclusion is that SMUF at equilibrium has a significantly different ion composition from that of freshly prepared SMUF. It does not represent salts in milk serum if allowed to reach equilibrium.

Acknowledgements

We thank Arie van den Berg and Peter Nobels from the Department of Soil Quality, Wageningen University, for performing ICP-AES measurement. We

would also like to thank MSc students Radhika Bongoni and Faridah for performing part of the experimental work.

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Chapter 4

Modelling ion composition in simulated milk ultrafiltrate (SMUF) II. Influence of pH, ionic strength and polyphosphates

This Chapter has been published as:

Gao, R., van Halsema, F. E. D., Temminghoff, E. J. M., van Leeuwen, H. P., van Valenberg, H. J. F., Eisner, M. D., & van Boekel, M. A. J. S. (2010). Modelling ion composition in simulated milk ultrafiltrate (SMUF) II. Influence of pH, ionic strength and polyphosphates. *Food Chemistry, 122*, 710-715.

Abstract

This study aims to determine whether the Dynamic Ion Speciation (DIS) model, as introduced in part I, can predict the ion composition in freshly prepared simulated milk ultrafiltrate (SMUF) under various conditions, e.g. pH, ionic strength and presence of various polyphosphates. Experiments were carried out in freshly prepared SMUF enriched with different salts. The model predictions were in satisfactory agreement with experimental results for all conditions. Moreover, specific ion effects were taken into account in the model, where sodium ions were not counted as equivalent to potassium ions due to differences in hydration properties. Polyphosphates, in particular sodium hexametaphosphate (SHMP), were well integrated into the DIS model with reasonable association constants and solubility products. The DIS model suggests that SHMP displays chain polyphosphate properties, which provides better understanding of its interaction with cations. The DIS model appears to be a robust and versatile tool to describe ion equilibria in SMUF.

Keywords: Ion equilibria, ion speciation model, pH, KCl, NaCl, polyphosphate, SMUF

4.1 Introduction

Ion equilibria, particularly the distribution of calcium and phosphate between the colloidal phase and serum phase of milk, play an important role in the conformation and stability of casein micelles (Fox & McSweeney, 1998; Holt, 1997; Walstra & Jenness, 1984). They have significant impact on the physicochemical properties of milk: namely heat stability, gelation induced by rennet and acid, fouling and sedimentation, thereby affecting the production and stability of products such as cheese, yoghurt and evaporated milk (Fox & McSweeney, 1998; Huppertz, 2007; Huppertz & Fox, 2006). Alterations of ion equilibria lead to significant changes in the micellar composition and structural integrity of casein micelles.

Acidification of milk solubilises the colloidal calcium phosphate and to some extent this can be accompanied by dissociation of casein from the micelle (Dalgleish & Law, 1989; Van Hooydonk, Hagedoorn & Boerrigter, 1986).

Increase in ionic strength by adding NaCl, has a significant effect on ion speciation in milk such as a decrease in pH, an increase in Ca^{2+} concentration, a decrease in the activity coefficient of ionic species and an increase in the hydration of casein micelles (Gaucheron, 2005; Huppertz & Fox, 2006). As a result, several properties of milk are changed, e.g. the rennet coagulation time of milk is increased (Famelart, Le Graet & Raulot, 1999; Van Hooydonk et al., 1986) and the ethanol stability is reduced (Huppertz & Fox, 2006). Up to now, little attention has been paid to the effect of increase in ionic strength by adding KCl since potassium ions are commonly regarded as equivalent to sodium ions. However, recent studies (Collins, 1997; Collins, Neilson & Enderby, 2007) show that potassium ions behave differently from sodium

ions in aqueous solutions due to differences in charge density and hydration properties. Such specific ion effect should be investigated in milk systems because adding NaCl or KCl may lead to significant differences in ion composition, such as Ca^{2+} activity and pH.

In addition, polyphosphates are widely used in dairy industry, particularly in the manufacturing of processed cheese products. Polyphosphates can influence the physicochemical properties of milk and dairy products because they can complex calcium and magnesium ions, leading to alteration of the ion equilibria and micellar structure. The regular polyphosphates used in dairy products are: tetrasodium pyrophosphate $Na_4P_2O_7$ (TSPP), sodium tripolyphosphate $Na_5P_3O_{10}$ (STPP) and sodium hexametaphosphate $(NaPO_3)_6$ (SHMP). These polyphosphates behave differently in milk systems in terms of calcium complexation, pH modification and direct interaction with the casein micelles (Shimp, 1983). Therefore, it is very important to choose the right phosphate for a given application. In spite of the wide application of polyphosphates, their ionic properties in multi-component systems have rarely been studied due to lack of knowledge of their ionic association constants and the solubility products of their salts. In particular, little is known of the molecular structure of SHMP and its ionization behaviour in solutions. Thus, it is of great practical importance to develop a comprehensive and predictive model of ion equilibria in milk under various conditions.

Previous work (Gao et al., 2010) has shown that the dynamic ion speciation (DIS) model adequately describes the ion equilibria in freshly prepared simulated milk ultrafiltrate (SMUF). The present investigation aims to validate the model in freshly prepared SMUF under conditions of changing

pH, ionic strength and added polyphosphates. Experiments were carried out to determine the ion composition in freshly prepared SMUF enriched with different electrolytes (HCl, NaCl, KCl, TSPP, STPP and SHMP). The work thus includes: (i) specific ion effects (e.g. Na^+ and K^+) in SMUF and their integration into the DIS model; (ii) the ionic behaviour of polyphosphates, particularly SHMP, and the concomitant quantification of their various ion association and precipitation reactions.

4.2 Materials and Methods

4.2.1 Experimental Setup

All experiments were carried out at 20 ± 1.0 ºC. Glassware was washed in phosphate-free detergent, soaked in 1.4 M nitric acid and rinsed in deionized water (Millipore, Amsterdam, The Netherlands). All chemicals were purchased from Fluka with purity 99.0 %, except for SHMP (purum) and STPP (≥ 98%). SMUF without lactose was prepared according to Jenness & Koops (1962).

4.2.1.1 Addition of HCl

The SMUF samples were acidified with 6M HCl from pH 6.6 to 4.5. The pH was measured using a pH electrode (Orion 8172BNWP, Thermo, Beverly, USA). Free Ca^{2+} activity was determined using a calcium ion selective electrode (Orion 97-20, Thermo, Beverly, USA). Free Ca^{2+} and Mg^{2+} ion concentrations were determined by Donnan Membrane Technique (DMT) as described by Gao et al. (2009). The DMT experiment was carried out in triplicate, and the calcium electrode measurement was repeated six times.

4.2.1.2 Addition of NaCl and KCl

NaCl or KCl was added to SMUF samples in the range between 0 and 0.3 mol/kg water. Same procedures for measuring pH, free Ca^{2+} activity and concentration were used as described in section 4.2.1.1. Each experiment was repeated six times.

4.2.1.3 Addition of polyphosphate

Three polyphosphates were used with various concentrations, as shown in **Table 1**. Adding TSPP and STPP resulted in precipitate formation in SMUF. The procedure of analyzing precipitates was described in the earlier work on the kinetics of calcium phosphate precipitation in SMUF (Gao et al., 2010).

Table 1 Characteristics of polyphosphates

Polyphosphate	Molecular formula	Concentration (w/w)
Tetrasodium pyrophosphate (TSPP)	$Na_4P_2O_7$	0 – 0.36 %
Sodium tripolyphosphate (STPP)	$Na_5P_3O_{10}$	0 – 0.50 %
Sodium hexametaphosphate (SHMP)	$(NaPO_3)_n{\cdot}Na_2O$	0 – 0.50 %

4.2.2 Determination of ionic calcium using a calcium ion selective electrode

Ionic calcium was determined using an Orion 97-20 ion-plus calcium electrode (Thermo, Beverly, USA). Calibration was carried out with freshly prepared standard solutions in the range between 10^{-4} and $3{\cdot}10^{-2}$ M $CaCl_2$. All the standard solutions were adjusted to ionic strength 0.09 M with KCl. The calibration curve plots potential (E) as a function of logarithm of calculated calcium activity. The time necessary for a stable reading was approximately 5 min, referring to a potential change less than 0.1mV per minute. Interference due to ions such as Na^+ and K^+ (section 4.3.2), are taken into account on the basis of the Nikolsky-Eisenman equation (Umezawa, Umezawa & Sato,

1995).

$$E = E^0 + \frac{RT}{z_{Ca^{2+}}F}\ln(a_{Ca} + k_{Ca,K}a_K{}^2 + k_{Ca,Na}a_{Na}{}^2 + ...) \quad (1)$$

where $z_{Ca^{2+}}$ is the charge of calcium ion; a_{Ca}, a_K and a_{Na} are the activities of Ca^{2+}, K^+ and Na^+ ion, respectively; $k_{Ca,\,K}$ and $k_{Ca,\,Na}$ are the selectivity coefficients of the calcium electrode with respect to K^+ and Na^+, which were determined according to the matched potential method (Umezawa et al., 1995) and were found to be 6.25×10^{-4} and 2.5×10^{-3}, respectively.

4.2.3 The model

The DIS model introduced in previous paper (Gao et al., 2010) was tested for its prediction of ion composition in freshly prepared SMUF at all the applied conditions described in section 4.2.1. This requires inclusion of another group of association constants of SHMP into the DIS model, as shown in **Table 2**. Incorporation of these pK_{ass} values did not affect the outcomes of the model as presented in the previous paper (Gao et al., 2010).

Table 2 pK_{ass} of cation-hexametaphosphate complexes used in the DIS model

Ion complex	pK_{ass}	Ion complex	pK_{ass}
$HP_6O_{19}^{7-}$	8.88 [d]	$NaP_6O_{19}^{7-}$	3.7 [b]
$H_2P_6O_{19}^{6-}$	6.7 [d]	$Na_2P_6O_{19}^{6-}$	2 [b]
$CaP_6O_{19}^{6-}$	10 [a]	$KP_6O_{19}^{7-}$	3.7 [c]
$Ca_2P_6O_{19}^{4-}$	7.5 [d]	$K_2P_6O_{19}^{6-}$	2 [d]
$MgP_6O_{19}^{6-}$	9 [a]	$NaHP_6O_{19}^{6-}$	2.9 [d]
$Mg_2P_6O_{19}^{4-}$	6.5 [d]	$KHP_6O_{19}^{6-}$	2.3 [d]

[a] Van Wazer et al. (1950)

[b] Kura et al. (1972)

[c] Kura et al. (1976)

[d] Estimated from model fitting in this research

4.3 Results and Discussion

4.3.1 Addition of HCl

With addition of HCl, anions such as citrate and phosphate are more strongly protonated, giving rise to the dissociation of ion complex species such as $CaCitrate^-$, $MgCitrate^-$ and $CaHPO_4$. On the other hand, addition of HCl leads to some increase in the formation of ion pairs such as $CaCl^+$. As a net result, the concentrations or activities of free Ca^{2+} and Mg^{2+} ions in SMUF increase as shown in **Figure 1**. The experimental data show general agreement with the concentrations or activities calculated by the DIS model. In particular, the DMT results for calcium were well predicted by the DIS model, but the DMT data for magnesium were slightly lower than the calculated concentration. This is probably due to some uncertainty in association constants of magnesium ion species, such as $MgCitrate^-$. In the range of pH between 5.20 and 6.63, the Ca-ISE results for calcium activity were somewhat lower than the DMT results and the model. The difference is probably caused by some weak interference from one or more components in SMUF (Holt, Dalgleish & Jenness, 1981).

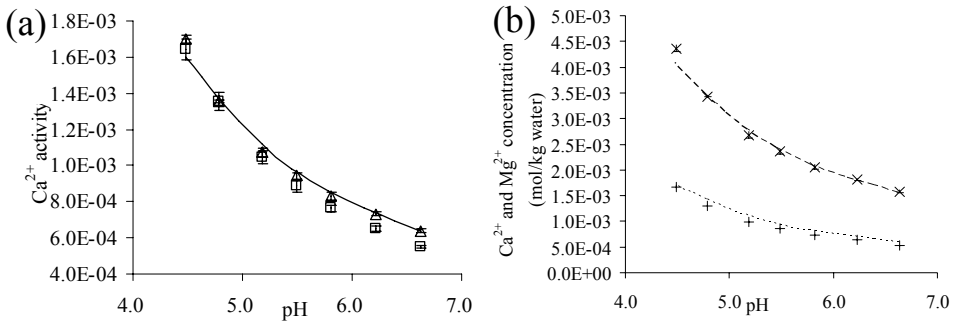

Figure 1 Effect of HCl addition on ion concentrations or activities in SMUF. (a) Comparison of calculated and experimental free Ca^{2+} activity in SMUF: (□) Ca-ISE; (△) DMT; (—) DIS model calculation. (b) Comparison of calculated and experimental free Ca^{2+} and Mg^{2+} concentration in SMUF: (×) and (+) free Ca^{2+} and Mg^{2+} concentration by DMT, respectively; (– –) and (---) free Ca^{2+} concentration by DIS model calculation, respectively. The DMT experiment was repeated in triplicate and the Ca-ISE measurement was repeated six times.

4.3.2 Addition of KCl and NaCl

Figure 2 shows that adding KCl or NaCl has several consequences for the ion composition in SMUF, such as variation of concentration and activity of cations, pH and ionic strength. There are significant differences between KCl and NaCl in their impact on ion composition of SMUF.

4.3.2.1 Effect of KCl and NaCl

Figure 2a shows that the Ca^{2+} activities, determined by DMT, tend to remain constant within experimental error throughout the concentration range of added KCl, as indicated by one-way ANOVA. The Ca^{2+} activities, calculated by the DIS model, were generally consistent with the DMT results. However,

the Ca^{2+} activities, measured by Ca-ISE, initially increased with the concentration of KCl and tend to become constant in the concentration range of KCl from 0.1 to 0.3mo /kg water. In general, the values of Ca^{2+} activities were in the order Ca-ISE ≤ DMT ≈ model. At lower concentration of KCl, the Ca-ISE results were significantly different from the DMT and model results. **Figure 2b** shows that the Ca^{2+} activities increased with addition of NaCl to SMUF. The DMT values and Ca-ISE values showed similar trend, but there were significant differences of the Ca^{2+} activities between the two methods at lower ionic strength, which appeared to generate problems similar to those found for KCl. Perhaps the calibration procedure on the basis of the Nikolsky-Eisenman equation (Eq. 1) is not working perfectly at lower ionic strength. At higher concentration of KCl or NaCl, the Ca^{2+} activities determined by Ca-ISE matched well with those by DMT and model.

Adding KCl or NaCl increases the ionic strength of SMUF and decreases ionic activity coefficients (**Figure 2f**). However, the concentrations of free Ca^{2+} or Mg^{2+} might increase due to increased competition of potassium in ion pair formation with citrate and others:

$$M + NCitrate^{-} \leftrightarrow MCitrate^{2-} + N$$

($M = K^{+}$ or Na^{+}; $N = Ca^{2+}$ or Mg^{2+}) (2)

Figure 2c shows that the calculated Ca^{2+} concentrations are in good agreement with the DMT results. The calculated Mg^{2+} concentrations differ from the DMT data, though the trends are similar (**Figure 2d**). The discrepancy could be caused by the association constants e.g. $MgCitrate^{-}$ and $CaCitrate^{-}$. By adjusting the association constant of $MgCitrate^{-}$, the calculated Mg^{2+} concentrations can be matched well with experimental results, albeit at the cost of some loss in quality of the modelled Ca^{2+} concentrations.

Considering the great importance of calcium ions, the DIS model was primarily optimised to match with experimental Ca^{2+} activities and concentrations.

Addition of KCl or NaCl significantly decreased the pH of SMUF (**Figure 2e**). The K^+ or Na^+ replaces the proton from $H_2PO_4^-$ to form ion pair $KHPO_4^-$ and releases H^+:

$$M + H_2PO_4^- \leftrightarrow MHPO_4^- + H^+ (M = K^+ \text{ or } Na^+) \quad (3)$$

The increase of H^+ concentration outweighs the decrease of the proton activity coefficient, resulting in a net decrease in the pH of SMUF. The calculated pH was well comparable to the experimental results.

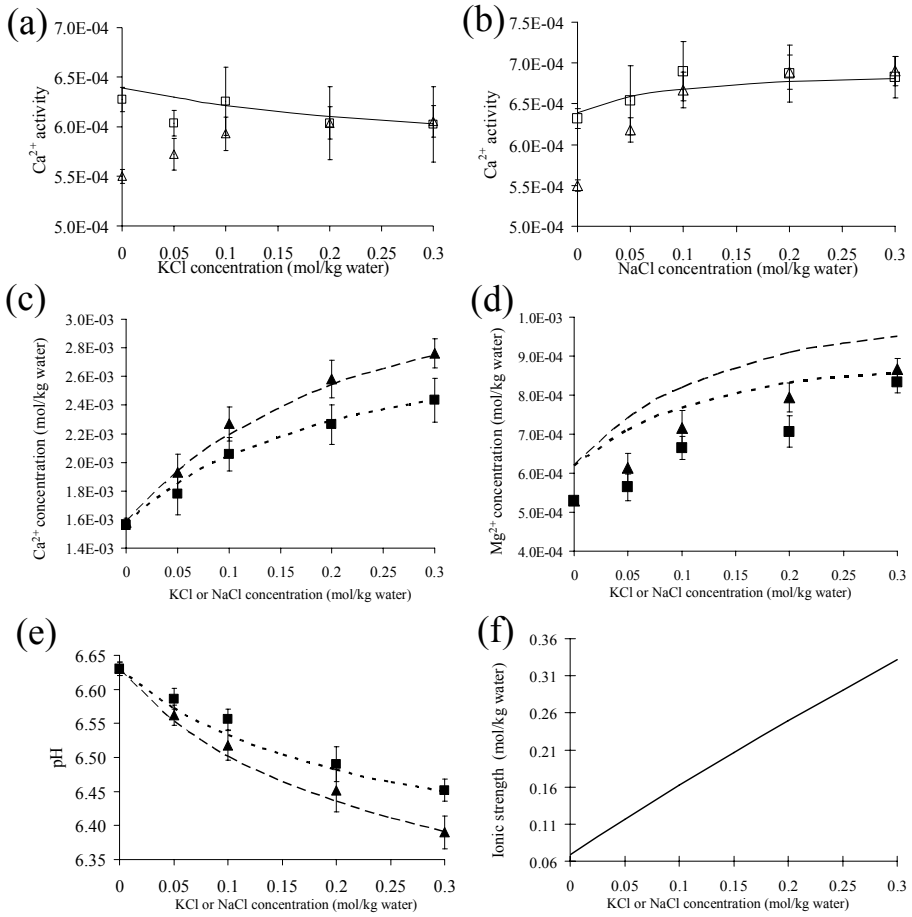

Figure 2 Effect of NaCl or KCl addition on ion composition in SMUF. (a) and (b) Comparison of calculated and experimental free Ca^{2+} activity in SMUF at different concentrations of KCl and NaCl, respectively: (□) DMT; (△) Ca-ISE; (—) DIS model calculation. (c), (d) and (e) Comparison of calculated and experimental free Ca^{2+} and Mg^{2+} ion concentration and pH in SMUF, respectively: (▲) and (■) represent experimental results for addition of NaCl and KCl, respectively; (– –) and (---) represent DIS model prediction for addition of NaCl and KCl, respectively. (f) The ionic strength of SMUF with additional NaCl or KCl. All data were collected six times.

4.3.2.2 Differences between KCl and NaCl

NaCl and KCl had significantly different effects on ion composition as expressed by pH, activity and concentration of Ca^{2+} and Mg^{2+} ions (**Figure 2**). The differences can be explained in terms of the dependence of hydration strength on charge density, as proposed by Collins (1997) and Collins et al. (2007). Briefly, their theory treats charge density as a central determinant of the structure and function of ion species in aqueous biological systems. It specifies the strength of water-water interactions and accurately describes simple ion-specific behaviour such as the tendency to form ion pairs. Small ions of high charge density (positive Jones-Dole viscosity B coefficient), named kosmotropes, bind water molecules tightly; on the other hand, large ions of low charge density (negative B), called chaotropes, bind water molecules relatively weakly compared to the strength of water-water interactions in bulk solution. This explains how oppositely charged ions preferentially interact to form ion pairs in solution. Specifically, oppositely charged ions in free solution form inner sphere ion pairs only when they have matched water affinities. Ion water affinity can be described by means of Jones-Dole viscosity B coefficient. **Table 3** shows that Na^+ and K^+ differ in their hydration features. Major anions in SMUF such as phosphate and citrate, are strongly hydrated due to their high charge densities. Na^+ is better matched to phosphate and citrate than is K^+ (**Table 3**), indicating that Na^+ interacts stronger with these anions to form ion pairs. In order to successfully model the impact of adding NaCl or KCl on the ion composition in SMUF, associated constants for ion pairs such as $NaCitrate^{2-}$ and $KCitrate^{2-}$ must be different from reported values (Holt et al., 1981). The latter values are only slightly different for $NaCitrate^{2-}$ and $KCitrate^{2-}$ and cannot explain the observed differences between Na^+ and K^+. Therefore, the association constants of $NaCitrate^{2-}$ and $KCitrate^{2-}$ were re-estimated to the new pK_{ass}

values of 1.9 and 1.53, respectively, as resulting from the best fit to experimental results.

Table 3. Jones-Dole viscosity B coefficients

Cations	Jones-Dole B	Anions	Jones-Dole B
H^+	0.068	PO_4^{3-}	0.59
Mg^{2+}	0.385	HPO_4^{2-}	0.382
Ca^{2+}	0.285	$Citrate^{3-}$	0.27
Li^+	0.15	SO_4^{2-}	0.208
Na^+	0.086	Cl^-	-0.007
K^+	-0.007	NO_3^-	-0.046

[a] H^+, HPO_4^{2-} and $Citrate^{3-}$ from (Chaplin, 2009) and the rest from (Robinson Jr, Strottmann & Stellwagen, 1981).

4.3.3 Addition of polyphosphates

4.3.3.1 Effect of TSPP and STPP

In SMUF, TSPP or STPP form insoluble Ca-polyphosphate compounds, resulting in a decrease in Ca^{2+} activity and an increase in pH (**Figure 3**). **Figure 3b** shows TSPP increases pH in SMUF more than does STPP because TSPP is more basic. Both the measured Ca^{2+} activity and the pH were predicted well by the DIS model.

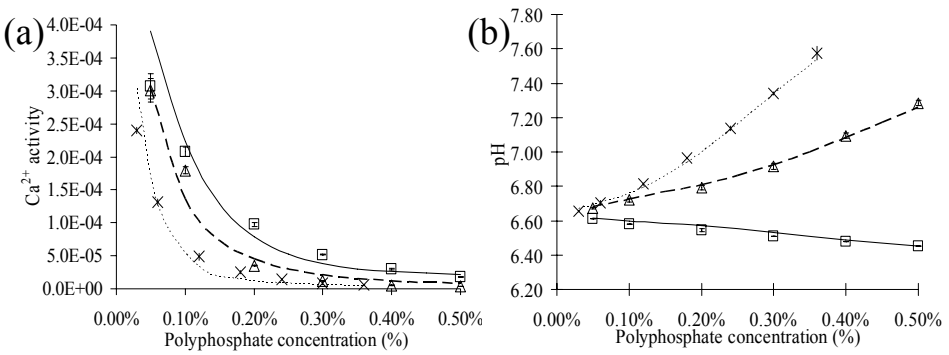

Figure 3 Effect of polyphosphates on ion composition in SMUF. (a) and (b) Comparison of calculated and experimental free Ca^{2+} activity and pH in SMUF, respectively: (□), (△) and (×) represent the experimental results for SHMP, STPP and TSPP, respectively; (—), (– –) and (---) represent the model prediction of the effect of SHMP, STPP and TSPP, respectively. All data were collected in triplicate.

TSPP and STPP differ in their characteristics of complexing and precipitating divalent cations. In SMUF, TSPP precipitates Ca^{2+} and Mg^{2+} ions, but STPP only precipitates Ca^{2+} ions. This finding was in accordance with the results by Van Wazer and Callis (1958). For the precipitates formed by TSPP, both the Ca/P molar ratio and Mg/P molar ratio were found to be around 1, representing the formation of calcium pyrophosphate ($Ca_2P_2O_7$) and magnesium pyrophosphate ($Mg_2P_2O_7$) **(Table 4)**. These precipitates are indeed predicted by the DIS model when applying the right solubility products. For the precipitates formed by STPP, the Ca/P molar ratio was approximately 0.66, suggesting the formation of dicalcium sodium tripolyphosphate ($Ca_2NaP_3O_{10}$) or dicalcium potassium tripolyphosphate ($Ca_2KP_3O_{10}$) (**Table 4**). Generally, STPP precipitates in two forms: $Ca_5(P_3O_{10})_2$ and $Ca_2NaP_3O_{10}$. According to Zhou and Carnali (2000),

$Ca_5(P_3O_{10})_2$ is formed for Ca/TPP ratios of 8 and 4 whereas $Ca_2NaP_3O_{10}$ is formed if the ratio is 2. This indicates that the higher the Ca/TPP ratio, the stronger is the preference for $Ca_5(P_3O_{10})_2$ precipitation. The ratio of Ca/TPP in SMUF varied from 0.66 to 6.6, but no $Ca_5(P_3O_{10})_2$ was found in the precipitates when the Ca/TPP ratio was higher than 2. This is not in agreement with the results reported by Zhou et al. (2000), which is probably due to differences in pH and the presence of other cations in SMUF and their different type of buffer system.

Table 4 Characteristics of different polyphosphates precipitates in SMUF

Polyphosphate	Molar ratio Ca/P	Molar ratio Mg/P	Precipitate
TSPP	1.01 (0.07) [a]	1.01 (0.07) [a]	$Ca_2P_2O_7$ & $Mg_2P_2O_7$
STPP	0.66 (0.03) [a]	no precipitate	$Ca_2NaP_3O_{10}$ & $Ca_2KP_3O_{10}$
SHMP	no precipitate	no precipitate	–

[a] n=3

4.3.3.2 Effect of SHMP

In contrast with TSPP and STPP, SHMP does not generate precipitation in SMUF. It does form soluble Ca/Mg-HMP complexes. Like other polyphosphates, SHMP significantly binds divalent metal ions, such as Ca^{2+}, leading to a decrease of Ca^{2+} activity (**Figure 3a**). Furthermore, SHMP decreases the pH in SMUF (**Figure 3b**), indicating the release of protons. De Kort, Minor, Snoeren, Van Hooijdonk and Van Der Linden (2009) and Vujicic, Batra and Deman (1967) indicated that the cause of pH drop is due to the replacement of hydrogen by calcium from the polyphosphates. However, according to De Kort et al. (2009), SHMP has been recognized as a cyclic compound with six metaphosphate monomers $(NaPO_3)_6$, as shown in **Figure 4a**. Given such molecular structure, SHMP would not release any protons;

instead, SHMP would rather bind protons to achieve protonation equilibrium between hexametaphosphate ($P_6O_{18}^{6-}$) and protonated hexametaphosphate $H_nP_6O_{18}^{n-6}$. Thus, SHMP with the cyclic structure could not explain the decrease of pH in SMUF. SHMP (purum) as supplied by Sigma-Aldrich is labelled as $(NaPO_3)_n{\cdot}Na_2O$, indicating SHMP is not cyclic. According to Zhang (1998), the actual molecular structure of SHMP is a chain polyphosphate (**Figure 4b**) and SHMP is regarded as a mixture of condensed phosphates, with a general formula $(Na, H)_{n+2}P_nO_{3n+1}$. Therefore, SHMP used in the experiments was considered to be a mixture of $Na_{n+2}P_nO_{3n+1}$ and $Na_nH_2P_nO_{3n+1}$.

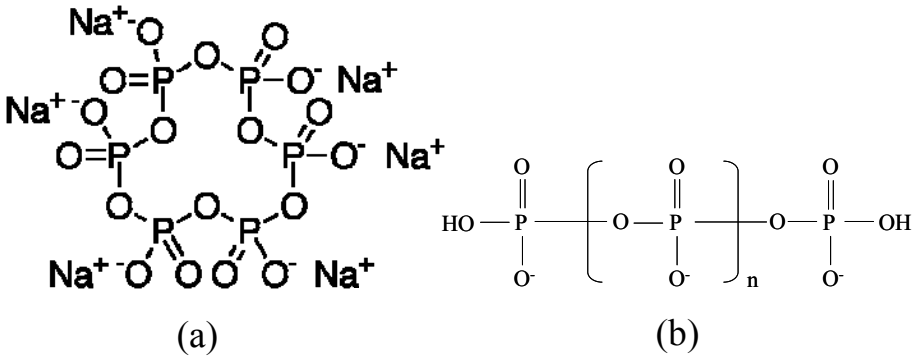

Figure 4 Molecular structures of sodium hexametaphosphate. (a) cyclic structure, (b) chain structure (Zhang, 1998).

In order to model the influence of SHMP on ion composition in SMUF, two assumptions were made: (i) SHMP contains six phosphorus atoms, which consists of $Na_8P_6O_{19}$ and $Na_6H_2P_6O_{19}$; (ii) The mass percentage of $Na_8P_6O_{19}$ and $Na_6H_2P_6O_{19}$ in SHMP was approximately 90 % and 10 % (w/w), respectively to obtain the best fit of the DIS model to experimental data.

Association constants of cation-hexametaphosphate complexes were employed as listed in **Table 2**. According to Van Wazer and Holst (1950), one strongly ionized hydrogen exists for every phosphorus atom and one weakly dissociated hydrogen was present at each end of a polyphosphate chain. **Figure 4b** shows that the SHMP molecule has two weak acid end groups. The two pK_{ass} values were estimated to be 8.88 and 6.70 for $HP_6O_{19}^{7-}$ and $H_2P_6O_{19}^{6-}$, respectively, based on the pK_{ass} values of STPP or TSPP. SHMP could complex two or more cations because of the available binding sites. However, due to lack of experimental data on pK_{ass} values of cation-hexametaphosphate complexes, some of the pK_{ass} values, such as, $Ca_2P_6O_{19}^{4-}$ and $Mg_2P_6O_{19}^{4-}$ were estimated for the best fit to the measured data. **Figures 3a** and **3b**, respectively, show that the calculated Ca^{2+} activity and pH are in good agreement with the experimental data. The slight differences are probably due to the uncertainties in some of the K_{ass} values adopted.

4.4 Conclusion

The dynamic ion speciation (DIS) model satisfactorily predicts ion composition in freshly prepared SMUF under most applied conditions at room temperature. However, deviations (within 15%) exist in Mg^{2+} concentration between predicted values and experimental values in the case of adding NaCl/KCl to SMUF. The DIS model distinguishes between Na^+ and K^+ taking into account their different hydration properties. Addition of sodium ions leads to higher Ca^{2+} activity and lower pH than does addition of potassium ions, and this has consequences for the stability of casein micelles. Therefore, it is important to realize these differences when using NaCl and KCl in dairy product development. Polyphosphates are integrated in the ion

speciation model, with realistic values for association constants for cation-polyphosphate complexes and solubility products for the precipitates. Polyphosphates have different calcium binding capacities and lead to different pH, implying the need to choose the appropriate polyphosphate for a specific dairy product. SHMP displays properties of a chain type polyphosphate with equilibrium between protonated and deprotonated forms. This feature is a necessary element in the proper understanding of its interaction with metal species in SMUF.

Acknowledgements

We are grateful to Arie van den Berg and Peter Nobels from the Department of Soil Quality, Wageningen University, for performing ICP-AES measurement. We would also like to thank MSc students Radhika Bongoni and Faridah for performing part of experimental work.

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Chapter 5

Effect of Disaccharides on Ion Properties in Milk-based Systems

This Chapter has been published as:
Gao, R., van Leeuwen, H. P., Temminghoff, E. J. M., van Valenberg, H. J. F., Eisner, M. D., & van Boekel, M. A. J. S. (2010). Effect of disaccharides on ion properties in milk-based systems. *Journal of Agricultural and Food Chemistry*, *58*(10), 6449-6457

Abstract

The mean spherical approximation (MSA) theory is used to explain the impact of sugars on ion properties in milk-based systems by taking into account electrostatic interactions and volume exclusion effects. This study first focuses on the changes in Ca^{2+} activity and pH in a solution consisting of $CaCl_2$, KCl and K_3Citrate, as a function of sucrose concentration. MSA model calculations were compared with experimental results and the model satisfactorily describes the ion properties. The excluded volume effects appear to account for a considerable increase in activity coefficient of the ions. This offers a sufficient explanation for the increase in Ca^{2+} activity and the decrease in pH in milk-based systems with added disaccharides. Besides, hydration of milk proteins seems to enhance ion pair formation in milk. All disaccharides lead to similar modification of the thermodynamic properties of milk-like systems, confirming that the observed effects are primarily due to volume exclusion effects.

Keywords: Sucrose, Lactose, Maltose, Trehalose, Calcium, pH, Activity coefficient, Milk, Mean spherical approximation

5.1 Introduction

Sugars are commonly added to food to contribute to physical-chemical, sensorial and microbial properties, such as controlling viscosity and texture, adding bulk, retaining moisture, reducing water activity and improve softness (Fennema, 1996; Lindsay, 1996). Studies show that sugars affect protein properties such as thermal stability, conformation, self- association, surface activity and hydration (Arakawa & Timasheff, 1982; Lee & Timasheff, 1981; Semenova, Antipova & Belyakova, 2002). Studies involving sugars and caseins suggest that addition of sugars leads to preferential hydration of the caseins (Mora-Gutierrez & Farrell H.M, 2000; Mora-Gutierrez, Kumosinski & Farrell Jr, 1997). The alteration of physico-chemical properties of casein micelles may have effects on the ionic equilibria, thereby changing the stability of milk products enriched with sugars, such as dairy desserts, and sweetened condensed milk which may contain sugar content up to 40 %. Therefore, it is of great practical importance and relevance to study the effects of addition of sugars on the ion properties in milk-based systems.

In milk, dynamic ion equilibria exist between the dissolved salts in milk serum and colloidal calcium phosphate in casein micelles. Ion equilibria play an essential role in the conformational stability of casein micelles (Fox & McSweeney, 1998). It has been found that addition of sucrose or glucose ranging from 0 to 6.5 mol/kg water, has an impact on thermodynamic properties of ions in solution, such as a decrease in pH in phosphate buffer solutions with a decrease of 0 and 0.7 pH unit (Bell & Labuza, 1992; Chuy & Bell, 2006) , and an increase in Ca^{2+} activity in milk (Geerts, Bekhof & Scherjon, 1983), but an explanation was not offered. The changes in Ca^{2+} activity and pH are crucial to dairy products during processing such as heat

stability and coagulation. As non-electrolytes, sugars are, in the first instance, not expected to affect the pH and ionic activities. The classical Debye–Hückel (DH) theory is not able to explain the observed phenomena. Addition of sugar can decrease the dielectric permittivity of water, and this causes a decrease in the activity coefficient of the ions in electrolyte solutions according to the DH theory, predicting a decrease in Ca^{2+} activity and H^{+} activity (Bell & Labuza, 1992; Geerts et al., 1983). This is thus inconsistent with the literature results. Moreover, it has been shown that the water activity reduction upon addition of sugars cannot explain the decrease in pH in the buffer solutions (Chuy & Bell, 2006). Additionally, other effects, such as whether addition of sugar affects ionic strength, still remain unclear.

A more recent theory, the mean spherical approximation (MSA), has been developed for the description of thermodynamic properties of electrolyte solutions (Fawcett, Tikanen & Henderson, 1997; Simonin, Blum & Turq, 1996; Tikanen & Fawcett, 1997). Compared to the DH model, the MSA approach is better suited to describe non-ideality of electrolyte solution in concentrated systems (ionic strength > 0.1M). In the MSA approach, all the solute species, charged or uncharged, have finite size (Fawcett et al., 1997). This allows one to take into account the excluded volume effects, e.g. via the hard sphere contribution, which reveals how space can be occupied by spheres (van Boekel, 2008). Also, the decrease in dielectric constant of the solution with increasing concentration of electrolyte or non-electrolyte is considered. This leads to an increase in the strength of ion-ion interactions with increasing ionic strength (Fawcett et al., 1997).

The aim of this paper is to investigate the applicability of the MSA theory to describe ion properties in milk-based systems enriched with disaccharides.

For this purpose, we developed a mathematical model based on the MSA to calculate the changes in Ca^{2+} activity and pH as a function of sucrose concentration in a multi-electrolyte solution containing $CaCl_2$, KCl and K_3Citrate. To validate the model, experiments were carried out in $CaCl_2$-KCl-K_3Citrate solutions with different sucrose concentrations. Subsequently, the MSA theory was used to qualitatively explain the increase in Ca^{2+} activity and decrease in pH in milk-based systems upon addition of sugars. In parallel, the influence of sugars on ion pair formation, conductivity, water activity and ion equilibria of milk were also studied and compared.

5.2 MSA theory

The MSA theory has been extensively applied to describe the thermodynamic properties of electrolytes and non-electrolytes (Fawcett & Tikanen, 1996; Simonin, 1997). MSA theory is used here to learn more about the molar activity coefficient of ion species i (y_i). Generally, the thermodynamic properties can be derived from the excess Helmholtz free energy ΔA^{EX}, which is made up of two contributions:

$$\Delta A^{EX} = \Delta A^{ES} + \Delta A^{HS} \quad (1)$$

ΔA^{ES}, the electrostatic part due to the ionic atmosphere, and ΔA^{HS}, the hard sphere part due to the finite size of the solute species.

The electrostatic contribution to the single ion activity coefficient can be written as follows (Tikanen & Fawcett, 1997)

$$\ln y_i^{ES} = -\frac{\beta e_0^2}{4\pi\varepsilon_0\varepsilon_S}\left[\frac{\Gamma z_i^2}{1+\Gamma d_i} + \varsigma \cdot d_i \cdot \left(\frac{2z_i - \varsigma d_i^2}{1+\Gamma d_i} + \frac{\varsigma d_i^2}{3}\right)\right] - \frac{\beta \Delta U^{ES}}{\varepsilon_S}\frac{\partial \varepsilon_S}{\partial \rho_i} \quad (2)$$

where e_0 represents the fundamental electronic charge, ε_S the relative

permittivity of solution, ε_0 the permittivity of free space, and $\beta = 1/kT$. The parameters d_i and ρ_i represent the effective diameter and number density of certain solute species, respectively. Γ is the MSA screening parameter which replaces the screening parameter κ in the Debye–Hückel theory. Γ becomes 0.5κ in the limit of zero ionic strength. The estimation of the model parameter ζ and screening parameter Γ are described by Tikanen and Fawcett (1997). ΔU^{ES} is the excess internal energy due to ion-ion interactions. Once the screening parameter Γ has been determined, the values of ζ and ΔU^{ES} are found by solving a series of equations described by Tikanen and Fawcett (1997).

In the $CaCl_2$-KCl-K_3Citrate solution with various sucrose contents, the only species changing the solution permittivity is sucrose. As a result, the value of $\partial\varepsilon_s/\partial\rho_i$ is assumed to be zero for estimation of $\ln y_i^{ES}$ for the calcium and hydrogen ion species. The solution permittivity depends on the concentration of the solutes. **Table 1** gives the tabulated permittivity of solution for different concentrations of sucrose (Malmberg & Maryott, 1950). Besides sucrose, the electrolytes $CaCl_2$, KCl and K_3Citrate contribute to a decrease in permittivity of the solution. This means that the $CaCl_2$-KCl-K_3Citrate solution enriched with sucrose should have lower permittivity than the aqueous solution containing only sucrose. The permittivity of the $CaCl_2$-KCl-K_3Citrate solution on the basis of the relationship (Tikanen & Fawcett, 1997) is:

$$\varepsilon_S = \varepsilon_{S0} - \delta_S c_e + b_S c_e^{3/2} \tag{3}$$

where ε_{S0} is the relative permittivity of pure water (78.45 at 25°C), ε_S is the relative permittivity of the solution, c_e is the electrolyte concentration, δ_S is the dielectric decrement for the given electrolyte and b_S is a parameter

describing the nonlinear part of this dependence. Due to lack of information about δ_S and b_S of calcium and citrate ions, we assume that the decrease in permittivity of pure water is completely caused by KCl. δ_S and b_S for KCl solution are 14.7 $mol^{-1}L$ and 3.0 $mol^{-3/2}L^{3/2}$, respectively (Tikanen & Fawcett, 1997). The concentration of KCl was estimated to be 0.073 mol/kg representing the same ionic strength of the $CaCl_2$-KCl-K_3Citrate solution, which corresponds to a decrease in permittivity of water by approximately 1.01. The resulting permittivities are shown in **Table 1**.

Table 1. Relative Permittivity (ε_S) at 25 °C of aqueous $CaCl_2$-KCl-K_3Citrate solution enriched with sucrose.

Sucrose (%) (w/w)	Sucrose (mol/L)	ε_S of sucrose solution [a]	Estimated ε_S of the $CaCl_2$-KCl-K_3Citrate-Sucrose solution [b]
0	0	78.45	77.44
10	0.304	76.19	75.18
20	0.632	73.65	72.64
30	0.988	70.86	69.85
40	1.376	67.72	66.71
50	1.798	64.2	63.19

[a] data were taken from Malmberg & Maryott (1950)

[b] used in the MSA model calculation and estimated from equation (3) as explained in the text

The unrestricted hard sphere contribution to $\ln y_i$ for unequally sized solute spheres is written as follows (Tikanen & Fawcett, 1997)

$$\ln y_i^{HS} = \left(\gamma_i - 1 - \frac{2\eta_i}{\eta} y_3\right) \cdot \ln(1-\eta) + \frac{\eta}{(1-\eta)^2} \cdot \left[3(1-\alpha_i) + \gamma_i + \frac{3\eta}{2}(\alpha_i - \beta_i - \gamma_i - 1)\right] + \frac{\eta}{(1-\eta)^3} \cdot \left\{\eta\left[5y_3 - \frac{9}{2}y_1 - 2 + \eta\left(\frac{3}{2}y_1 - 3y_2 - 4y_3 + 1\right)\right] - 2y_3 + 4\right\} \quad (4)$$

$$\eta_i = \frac{\pi}{6}\rho d_i^3, \qquad \eta = \sum_i \frac{\rho_i}{\rho}\eta_i, \qquad \rho = \sum_i \rho_i$$

The parameters introduced in equation (4) were described by Tikanen and Fawcett (1997).

Combination of equation (2) and (4) yields the expression for the single ion molar activity coefficient

$$\ln y_i = \ln y_i^{ES} + \ln y_i^{HS} \tag{5}$$

In addition, cations and anions can associate to form ion pairs, which contribute to the single ion activity coefficient. Only 1:1 ion pair formation is taken into account. The association constant (K_{ass}) can be written as

$$K_{ass} = \frac{a_{ip}}{a_+ \cdot a_-} = \frac{(1-\alpha)}{\alpha^2 c} \cdot \frac{y_{ip}}{y_+ \cdot y_-} \tag{6}$$

where a_{ip}, a_+ and a_- are the activity of ion pair, cation and anion, respectively. (1 - α) is the fraction of cations forming ion pairs, c is the molar concentration of the electrolyte, y_+, y_- and y_{ip} represent molar activity coefficient of cation, anion and ion pair, respectively.

The MSA model for the description of the thermodynamic properties of electrolyte solutions has been developed at the so-called McMillan-Mayer (MM) level (Simonin, 1999). Briefly, the MM framework considers the solvent as a continuum, not as consisting of discrete particles/molecules, and the solvent is characterized only by its permittivity (van Boekel, 2008). That means that only solute-solute interaction potentials are considered in the MM framework. The ion activity coefficients calculated by MSA cannot be directly compared to experimental values which are described in the Lewis-Randall (LR) framework unless a conversion is made from MM level to LR level. The LR description of a solution considers the excess Gibbs energy, the energy state function defined with temperature (T), pressure (P),

number of moles (n) as the independent variables (which are the three natural variables in a chemical experiment) (van Boekel, 2008). The effect of the conversion was negligible at relatively low concentrations, but it became significant in highly concentrated electrolyte solutions, typically above 1-2 mol/kg water (Simonin, 1996). In this study, the concentrations of the salts were far below 1 mol/kg water. However, there was a large amount of sucrose present in some of the systems. Therefore, the conversion of ion activity coefficient would be needed so that the modeled activity coefficient can be compared to the experimental results. The way to do this conversion has been described by Simonin (1997). Briefly, the first step is to convert the molar activity coefficient from the MM to LR level:

$$\ln a_{\mathrm{W}} = -\phi^{(\mathrm{LR})} M_{\mathrm{W}} \cdot \sum_{\mathrm{j}=1}^{\mathrm{j}=\mathrm{i}} m_{\mathrm{j}} \tag{7}$$

where $\phi^{(\mathrm{LR})}$ is the osmotic coefficient at the LR level. Equation (7) is taken from the literature (Blandamer, Engberts, Gleeson & Reis, 2005). a_{w} is the water activity. Water activity data at different concentrations of sucrose were obtained from Robinson and Stokes (2002). M_{w} is the molecular weight of water and m_{j} is the molal concentration of the solute.

$$\phi^{(\mathrm{LR})} = \phi^{(\mathrm{MM})} \cdot (1 - C \cdot V_{\pm}) \tag{8}$$

$$\ln y_{\mathrm{i}}^{(\mathrm{LR})} = \ln y_{\mathrm{i}}^{(\mathrm{MM})} - \phi^{(\mathrm{MM})} \cdot C \cdot V_{\mathrm{i}} \tag{9}$$

where $\phi^{(\mathrm{MM})}$ is the osmotic coefficients at the MM level, respectively. C is the total solute concentration (mol/L). $V_{\pm}$ is the mean partial molal volume of the solute, and V_{i} is the partial molal volume of species i. $y_{\mathrm{i}}^{(\mathrm{MM})}$ and $y_{\mathrm{i}}^{(\mathrm{LR})}$ are the molar activity coefficients at the MM and LR level, respectively. $y_{\mathrm{i}}^{(\mathrm{MM})}$ is calculated by eq (5).

The next step is to convert the molar activity coefficient to molal activity coefficient within the LR level:

$$y_i^{(LR)} = \gamma_i^{(LR)} \cdot V \cdot d_W \quad (10)$$

$\gamma_i^{(LR)}$ is the molal activity coefficient of species i at LR level. V is the volume of the solution per mass solvent in the LR system. d_W is the density of water. The obtained values of $\gamma_i^{(LR)}$ for Ca^{2+} ions were compared to the experimental activity coefficients of SMUF and skim milk enriched with sugars.

5.3 Materials and Methods

5.3.1 Materials

All the salts (> 99.5% purity) used were obtained from Fluka (The Netherlands). Sucrose, maltose monohydrate and trehalose dihydrate (HPLC grade) were obtained from Sigma (The Netherlands). Lactose monohydrate (HPLC grade) was purchased from Merck (Germany). The low heat pasteurized skim milk powder (Nilac) was obtained from NIZO (The Netherlands).

5.3.2 Experimental Setup

Table 2 shows the experimental set-up. Three systems of increasing complexity were employed: (i) a solution consisting of $CaCl_2$ (5 mM), KCl (55 mM) and K_3Citrate (4 mM) (ii) lactose-free simulated milk ultrafiltrate (SMUF) (iii) reconstituted skim milk. SMUF was prepared according to Jenness and Koops (1962). The reconstituted skim milk was prepared from low heat skim milk powder as described by Gao et al. (2009). Four disaccharides were separately added to each system except for the $CaCl_2$-KCl-K_3Citrate system where only sucrose was used. Prior to analyses,

the systems with added lactose and maltose were kept at 25 °C for 24 hours to allow the reducing sugars reaching their mutarotation equilibrium. The systems with added sucrose and trehalose were directly measured. Physico-chemical properties, pH, Ca^{2+} activity, water activity, conductivity and Ca^{2+} concentration of selected samples were measured as described in the methods section. Moreover, the MSA model was used to calculate the pH and Ca^{2+} activity in the $CaCl_2$-KCl-K_3Citrate solution with various sucrose contents.

Table 2. Overview of the experimentally studied systems

		Analyses [a]			DMT [c]		
Sugars	% (w/w)	$CaCl_2$-KCl-K_3Citrate [b]	SMUF	Skim Milk	$CaCl_2$-KCl-K_3Citrate	SMUF	Skim Milk
None	0	x	x	x	x	x	x
Sucrose	5	- [d]	x	x	-	-	-
	10	x	x	x	-	x	x
	20	x	x	x	x	x	x
	30	x	x	x	-	x	x
	40	x	x	x	-	-	-
	50	x	-	-	-	-	-
Lactose	5	-	x	x	-	-	-
	10	-	x	x	-	x	x
	15	-	x	x	-	-	-
Maltose	5	-	x	x	-	-	-
	10	-	x	x	-	x	x
	15	-	x	x	-	-	-
	20	-	x	x	-	-	-
Trehalose	5	-	x	-	-	-	-
	10	-	x	-	-	x	-
	15	-	x	-	-	-	-

[a] pH, Ca^{2+} activity (Ca-ISE), conductivity and water activity measurements

[b] The MSA theory was applied to calculate the effect of sucrose on Ca^{2+} and H^+ ion activity in the system $CaCl_2$-KCl-K_3Citrate.

[c] Donnan membrane technique (DMT) was used to determine the concentrations of free Ca^{2+} and Mg^{2+} ions.

[d] - not measured

5.3.3 Methods

5.3.3.1 pH measurement

The pH values of selected samples were measured at 25 °C using a pH meter (Orion 720A+, Thermo, Beverly, USA) equipped with a Ross pH electrode (Orion 8172BNWP, Thermo, Beverly, USA). The pH electrode was calibrated at 25 °C using two buffer solutions (Thermo, USA) having pH values of 4.00 and 7.00.

5.3.3.2 Calcium activity measurement

Ionic calcium activity was determined as described by Gao et al. (2009) using an Orion 720A+ meter equipped with an Orion 9720 calcium ion selective electrode (Ca-ISE) (Thermo, Beverly, USA).

5.3.3.3 Donnan membrane technique (DMT)

The Donnan membrane technique (DMT) was applied to determine the concentrations of free Ca^{2+} and Mg^{2+} ions in the selected samples (**Table 2**). The details of the DMT methodology were described by Gao et al. (2009) and Temminghoff et al. (2000). Briefly, the sample solution, also named the donor, is separated from the acceptor solution by a cation exchange membrane (BDH Laboratory Supplies, Poole, UK). The negative electric potential of the membrane allows cations to pass through and restricts anions

from crossing the membrane. Within certain time, Donnan equilibrium is reached for the free metal ion activities between donor and acceptor. If both sides have the same ionic strength, the free metal concentration in donor is equal to it in acceptor. In this research, the acceptor solution had the same amount and type of sugar and the same ionic strength as the donor solution did. This eliminated the differences in osmotic pressure and excluded volume between the donor and the acceptor. Samples taken from both sides were measured by inductively coupled plasma atomic emission spectrometer (ICP-AES) (Varian, Mulgrave, Australia).

5.3.3.4 Conductivity

Conductivities were measured to check for ion pair formation upon addition of sugar. Sugar molecules can bind free water molecules, leading to a decrease in water activity. Addition of sugar molecules to electrolyte solution may even cause dehydration of ions due to strong hydration of sugar, implying that ion pair formation may be promoted. This would then affect the conductivity of the electrolyte solution.

Conductivity (G) can be expressed as follows (Atkins & de Paula, 2006)

$$G = zuvcF \tag{11}$$

$$u = \frac{ze_0}{6\pi\eta_s a} \tag{12}$$

By combining equations (11) and (12),

$$G = \frac{z^2 e_0 vcF}{6\pi r} \times \frac{1}{\eta_s} \tag{13}$$

where z, the charge of the ion, u, the mobility of the ion, νc, the molar concentrations of the ion, F, Faraday's constant, r is the radius of ion and η_s is the viscosity of solution.

Adding sugar increases the viscosity of electrolyte solutions, which in itself causes a decrease in conductivity. Equation 13 is therefore split into two parts. The left part accounts for the ion pair formation and the right part represents the influence of viscosity on conductivity. When a linear relationship is obtained between G and $1/\eta_s$, the left part apparently remains constant, meaning that ion pair formation would be insignificant. The viscosity data of a series of sucrose solutions were obtained from literature (Weast, Astle & Beyer, 1987). The viscosity of milk samples enriched with sucrose was measured as described in the section of viscosity measurement.

The conductivities of selected samples were measured using a conductivity meter (WTW Inolab Cond Level 2, Weilheim, Germany) equipped with a conductivity electrode (WTW TetraCon 325, Weilheim, Germany). The conductivity probe was calibrated at 25 °C using a 0.01 M KCl solution having conductivity values ranging between 1411 and 1423 μS/cm.

5.3.3.5 Viscosity

The viscosity of skim milk samples enriched with sucrose was determined by using a Anton Paar MCR 300 rheometer and a double gap geometry (DG 26,7 mm). Shear rate ranged from 0.01 - 500 s^{-1}. The viscosity was determined between the shear rate 10 and 500 s^{-1}. The measurements were made in duplicate. The temperature of the samples was 25 °C.

5.3.3.6 Water activity

The water activity of selected samples (**Table 2**) was measured using an AquaLab CX3 (Decagon Devices, Washington, USA).

5.3.3.7 Ultracentrifugation

The skim milk samples enriched with sugars were ultracentrifuged (at 100 000 *g*, 25 °C for 1 hour) in a Beckman L-60 ultracentrifuge with a 70 Ti rotor (Beckman Instruments Inc, California, USA). The concentrations of total Ca, Mg, K, Na and P in milk serum were measured by ICP-AES (Varian, Mulgrave, Australia).

5.4 Results and discussion

5.4.1 Addition of sucrose to $CaCl_2$-KCl-K_3Citrate solution

Figure 1a and **1c** show that the addition of sucrose (0 – 50 % w/w) causes a considerable increase in Ca^{2+} and H^+ ion activities, respectively, in the $CaCl_2$-KCl-K_3Citrate solution. The Ca^{2+} activity and pH calculated by the MSA model were in general agreement with the experimental data after using the values of the diameters of cation (d_{cation}), anion (d_{anion}), ion pair (d_{ip}) and sucrose ($d_{sucrose}$) and the association constants (K_{ass}) in **Table 3** and the dielectric permittivity data in **Table 1**. The estimated diameters of the species were chosen on the basis of the agreement between model prediction and experimental results. The diameters for individual ions and ion pairs are in fair agreement with the values from literatures (Corti, 1987; Pazuki & Arabgol, 2006; Simonin, 1999; Tikanen & Fawcett, 1997). However, differences in Ca^{2+} activity and pH between calculation and measurement became larger at higher sucrose concentrations (**Figure 1a and 1c**). Ions may behave far more non-ideally in such highly concentrated systems so that the MSA model may also be no longer applicable. The diameter of hydrated cations decreases as sucrose concentration increases, and may decrease considerably in the high sucrose concentration solutions. Such changes in cation diameter were not taken into account in the MSA model due to the

lack of diameter data for cations in sucrose solutions.

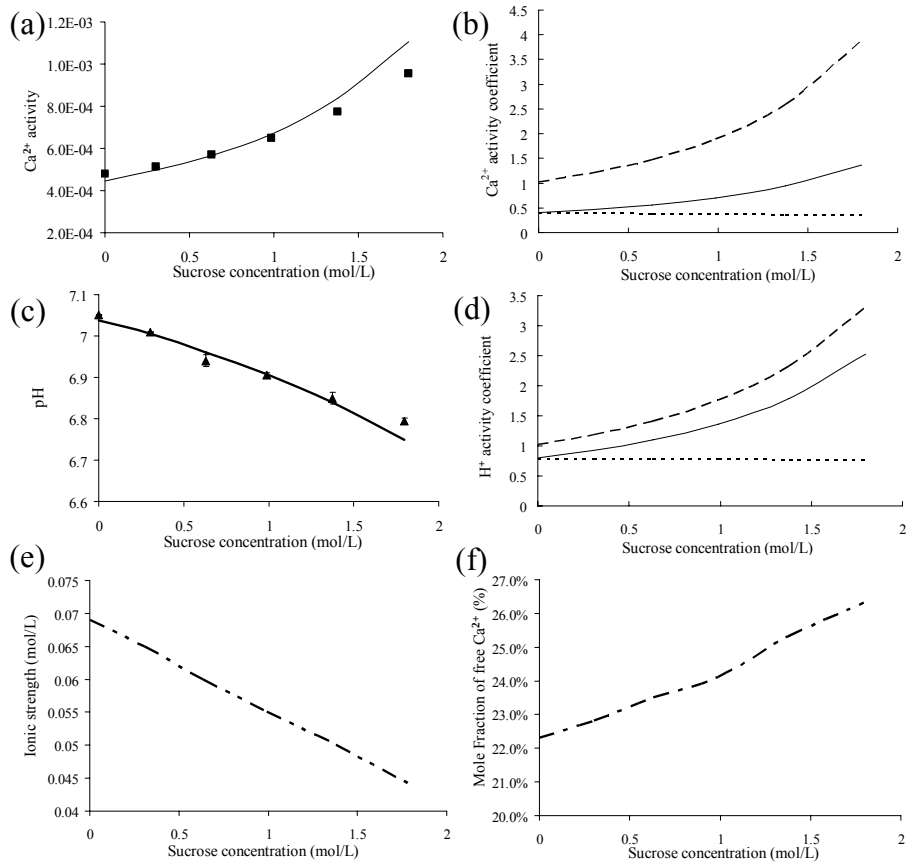

Figure 1. Influence of sucrose on ionic properties in $CaCl_2$-KCl-K_3Citrate system. (a) and (c) represent Ca^{2+} activity and pH, respectively, as a function of sucrose concentration (■) measured Ca^{2+} activity by Ca-ISE, (—) calculated Ca^{2+} activity by MSA model, pH as a function of sucrose concentration (▲) measured pH, (▬) calculated pH by MSA model. (b) and (d) represent the calculated Ca^{2+} and H^+ activity coefficient by MSA model, respectively. (---), (– –) and (—) represent activity coefficient of the electrostatic part, the hard sphere part and the overall, respectively. (e) represents the calculated ionic strength (mol/L). (f) represents the calculated mole fraction of free Ca^{2+} as a function of

sucrose concentration by the MSA model.

Table 3. Values of the association constants K_{ass} and the diameters of cation (d_{cation}), anion (d_{anion}), ion pair (d_{ip}) and sucrose ($d_{sucrose}$) employed in the MSA model to calculate Ca^{2+} activity and pH in the $CaCl_2$-KCl-K_3Citrate solution.

Reactants	K_{ass} [a]	d_{cation} (pm)	d_{anion} (pm)	d_{ip} (pm)	$d_{sucrose}$ (pm)
K^+, Cl^-	1	350	350	350	-
Ca^{2+}, Cl^-	7	480	350	415	-
Ca^{2+}, $Citrate^{3-}$	$1.66 \cdot 10^5$	480	400	440	-
H^+, $Citrate^{3-}$	$2.51 \cdot 10^6$	400	400	400	-
Sucrose	-	-	-	-	700

[a] the association constants were taken from literature (Gao et al., 2010a)

The changes in Ca^{2+} activity and pH rely on the free ion concentrations and single ion activity coefficients. **Figure 1b and 1d** reveal that the increase in activity coefficient is the main contribution to the increase in Ca^{2+} or H^+ activity in the $CaCl_2$-KCl-K_3Citrate solution enriched with sucrose. The addition of sucrose contributes to the changes in ion activity coefficient in two ways. Firstly, on the one hand, the decrease in permittivity of the solution causes a decrease in the ion activity coefficient due to enhanced electrostatic interaction. On the other hand, the calculated ionic strength decreased from 0.069 to 0.044 mol/L due to an increase in volume (**Figure 1e**), which enlarged the ion activity coefficient. **Figure 1b and 1**d show the summation of the two effects. The calculated y_{Ca}^{ES} and y_H^{ES} decreased from 0.390 to 0.355 and from 0.782 to 0.764, respectively. Secondly, the system is occupied by many sucrose molecules, leading to an increase in excluded volume effect. This accounts for the considerable departure from ideality of the ions in the concentrated sucrose solutions. **Figure 1b and 1d** show that the calculated y_{Ca}^{HS} and y_H^{HS} remarkably increase from 1.025 to 3.859 and from 1.022 to

3.298, respectively, as a function of sucrose concentration. By combining the electrostatic and hard sphere contribution, it turns out that the single ion activity coefficient still increases significantly, for y_{Ca} from 0.400 to 1.369, and for y_H from 0.799 to 2.519 (**Figure 1b and 1d**). Additionally, the molar fraction of free Ca^{2+} ions was calculated by the MSA model (**Figure 1f**) and it was found to slightly increase from 22 % to 26 % throughout the range of sucrose concentrations. Consequently, the increase in activity coefficient, particularly the excluded volume effect, is the main reason for the increase in ion activity in the $CaCl_2$-KCl-K_3Citrate solution enriched with sucrose. The MSA theory appears to offer a satisfactory explanation for the observed ion behaviors in electrolyte solutions at very high content of sucrose. In addition, the effect of using the MM-to-LR conversion (Eq.(7)-(10)), that is from $y_{Ca}^{(MM)}$ to $\gamma_{Ca}^{(LR)}$, was examined. **Table 4** shows that the difference between $y_{Ca}^{(MM)}$ and $\gamma_{Ca}^{(LR)}$ increases as the concentration of sucrose increases，which indicates that the effect of this conversion is not negligible, particularly at very high sucrose contents. Thus, the values of obtained $\gamma_{Ca}^{(LR)}$ were used to compare with experimental molal Ca^{2+} activity coefficient of milk-based systems in the presence of sugars. In the following section, MSA theory was used to qualitatively explain the ion properties in milk-based systems upon addition of sugars.

Table 4. Ca^{2+} activity coefficients at the MM and LR level in $CaCl_2$-KCl-K_3Citrate solution enriched with sucrose using equations (7) – (10).

Sucrose (%) (w/w)	$y_{Ca}^{(MM)}$	$y_{Ca}^{(LR)}$	$\gamma_{Ca}^{(LR)}$
10	0.47	0.46	0.43
20	0.56	0.55	0.48
30	0.70	0.69	0.54
40	0.94	0.91	0.64

5.4.2 Addition of sucrose to SMUF and skim milk

5.4.2.1 Excluded volume effect

Like in the $CaCl_2$-KCl-K_3Citrate system, the addition of sucrose increases the Ca^{2+} and H^+ ion activities in SMUF and skim milk (**Figure 2b and 2e**). The Ca^{2+} activity coefficient, which was calculated from the experimental free Ca^{2+} concentration and activity, increased considerably throughout the range of sucrose concentration (**Figure 2c**). It stands to reason that the excluded volume effect accounted for the increase in Ca^{2+} and H^+ activities. The increase in Ca^{2+} activity coefficient in skim milk was larger than it was in SMUF. This is probably because skim milk contains proteins and lactose, which account for stronger excluded volume effects.

However, the experimentally derived values of Ca^{2+} activity coefficients of SMUF and skim milk in the absence of sucrose were far too low compared to the value commonly calculated by Davies equation, which is 0.40 for both SMUF and skim milk (**Table 5**). On the one hand, we speculate that a systematic deviation is generated by the Ca-ISE methodology at lower ionic strength (I < 0.09 mol/kg water) for the Ca^{2+} activity measurement, since these results are consistent with earlier observations (Gao et al., 2009; Gao et al., 2010b). On the other hand, the calculated Ca activity coefficient by the Davies equation may overestimate the actual value in a multi-component solution (Butler, 1968). We are in the process of analyzing the causes of the large differences in individual Ca ion activity coefficient between experimental and calculated results and will discuss the issue in a separate publication.

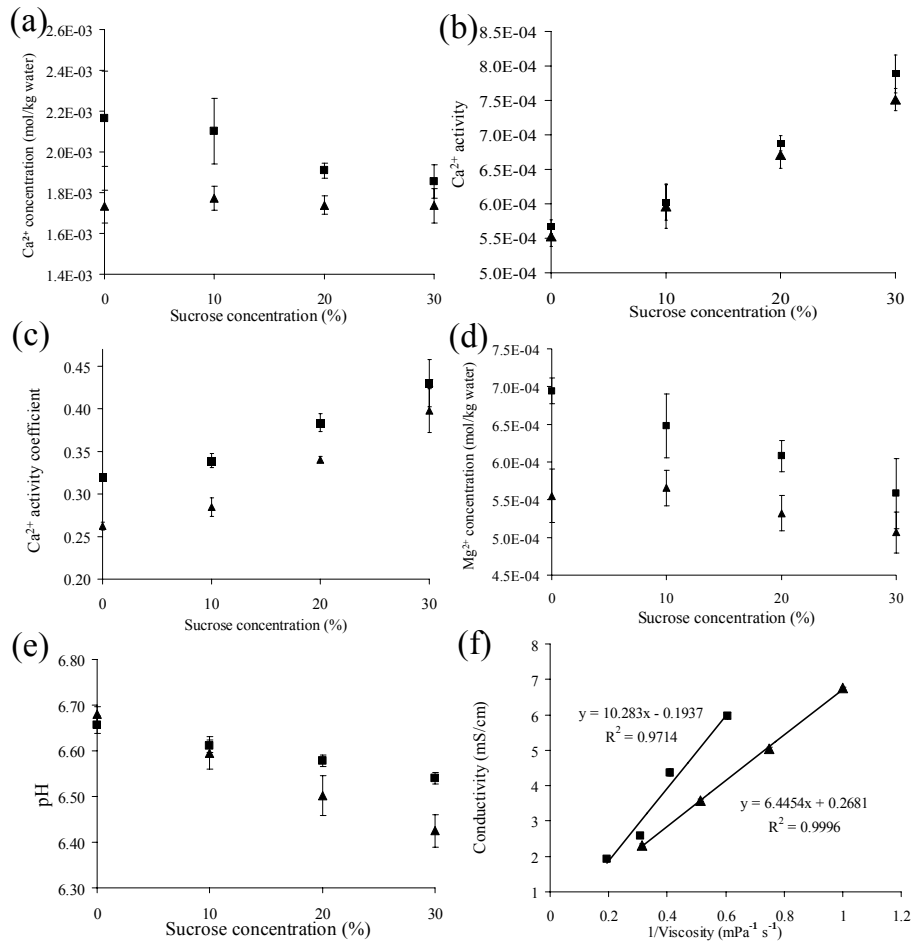

Figure 2. Influence of sucrose on ion properties, (▲) and (■) represent SMUF and Skim milk, respectively. (a) Ca^{2+} concentration measured by DMT, (b) Ca^{2+} activity measured by Ca-ISE, (c) Ca^{2+} activity coefficient calculated from experimental data, (d) Mg^{2+} concentration measured by DMT and (e) pH in SMUF and skim milk measured by pH electrode. (f) Relation between conductivity and viscosity at different sucrose concentrations.

A correction was made to adjust the experimental Ca^{2+} activity coefficients in SMUF according to the method described by Geerts et al. (1983). Briefly, the

experimental Ca^{2+} activities were plotted as a function of the added sugar concentration. The Ca^{2+} activities were found to correlate linearly with the amount of sugar added (**Table 6**). Since the free Ca^{2+} concentration of SMUF is not influenced by the additional sugar (**Figure 2a and Table 8**), this indicates that the increase in Ca^{2+} activity coefficient is the same as the increase in Ca^{2+} activity after addition of sugar. That is, the ratio of Ca^{2+} activity coefficient in the presence and absence of sugar should be equal. **Table 6** gives the adjusted Ca^{2+} activity coefficients in the presence of sugars in SMUF. Interestingly, the adjusted Ca^{2+} activity coefficients in SMUF are nearly same as the calculated values ($\gamma_{Ca}^{(LR)}$) of the $CaCl_2$-KCl-K_3Citrate solution enriched with sucrose by the MSA model (**Table 4**). This implies that the approach used by Geerts et al. (1983) gives good prediction in Ca^{2+} activity coefficients in electrolyte solutions. For skim milk, the Ca^{2+} activity also increased linearly with the content of sugar (**Table 6**). However, the free Ca^{2+} concentration decreased with the added sugar, e.g. sucrose (**Figure 2a**). Thus, the Ca^{2+} activity coefficients (**Table 6**) need to take into account the change in the Ca^{2+} concentration and can be calculated as follows:

$$\frac{a_{Ca2+,(S)}}{a_{Ca2+,(B)}} = \frac{m_{Ca2+,(S)}}{m_{Ca2+,(B)}} \cdot \frac{\gamma_{Ca2+,(S)}}{\gamma_{Ca2+,(B)}} \tag{14}$$

Equation 14 can be rearranged as follows:

$$\gamma_{Ca^{2+},(S)} = \gamma_{Ca^{2+},(B)} \cdot \frac{a_{Ca^{2+},(S)}}{a_{Ca^{2+},(B)}} \cdot \frac{m_{Ca^{2+},(B)}}{m_{Ca^{2+},(S)}} \tag{15}$$

$\gamma_{Ca^{2+},(S)}$ represents the adjusted molal Ca^{2+} activity coefficient at certain sugar concentration. $\gamma_{Ca^{2+},(B)}$ is molal Ca^{2+} activity coefficient of milk in the absence of sugar (**Table 5**). $m_{Ca^{2+},(S)}$ and $a_{Ca^{2+},(S)}$ are the molal Ca^{2+} concentration and the calculated Ca^{2+} activity of milk in the presence of sugar, respectively (**Table 6 and 8**). $m_{Ca^{2+},(B)}$ and $a_{Ca^{2+},(B)}$ are the Ca^{2+} concentration

and the calculated Ca^{2+} activity of milk in the absence of sugar, respectively (**Table 6 and 8**). For the milk samples without measuring the Ca^{2+} concentrations, the same correction method was used as described for SMUF.

Table 5. Ca^{2+} activity coefficients (γ_{Ca}^{2+}) (SD)[a] of SMUF and skim milk in the absence of sugar

	SMUF [b]	Skim milk [b]	Theoretical [c]
γ_{Ca}^{2+}	0.32 (0.00)	0.26 (0.01)	0.40

[a] n=2

[b] Activity coefficients were calculated from the Ca^{2+} activity (Ca-ISE method) (**Table 6**) and Ca^{2+} concentration (DMT method) (**Table 8**)

[c] Value was calculated by the Davies equation.

Table 6. Effect of added sugar on the Ca^{2+} activity and the Ca^{2+} activity coefficient ($\gamma_{Ca^{2+},(S)}$) (in terms of molality) of SMUF and Skim milk

		Ca^{2+} activity (SD) [a]								
		SMUF				Skim Milk				Fit Equations [f]
Sugars	% (w/w)	Measured [b]	Calculated [c]	Ratio [d]	$\gamma_{Ca^{2+},(S)}$ [e]	Measured [b]	Calculated [c]	Ratio [d]	$\gamma_{Ca^{2+},(S)}$ [e]	$a_{Ca^{2+}} = b \cdot s + c$
None	0	0.55 (0.01)	0.55	1.000	0.40	0.57 (0.01)	0.57	1.000	0.40	
Sucrose	5	0.57 (0.02)	-	-	-	0.58 (0.02)	-	-	-	$a_{Ca^{2+}} = 0.0007 \cdot s + 0.00055$ (SMUF)
	10	0.60 (0.03)	0.62	1.122	0.45	0.60 (0.03)	0.64	1.129	0.46	$R^2 = 0.984$
	20	0.67 (0.02)	0.69	1.249	0.50	0.69 (0.02)	0.71	1.252	0.57	$a_{Ca^{2+}} = 0.0007 \cdot s + 0.00057$ (skim milk)
	30	0.75 (0.02)	0.76	1.375	0.55	0.79 (0.03)	0.78	1.376	0.64	$R^2 = 0.961$
	40	0.92 (0.01)	-	-	-	0.94 (0.05)	-	-	-	
Lactose	5	0.57 (0.03)	0.59	1.059	0.42	0.58 (0.00)	-	1.059	0.42	$a_{Ca^{2+}} = 0.0007 \cdot s + 0.00055$ (SMUF)
	10	0.61 (0.03)	0.62	1.122	0.45	0.60 (0.02)	-	1.122	0.44	$R^2 = 0.970$
	15	0.65 (0.02)	0.66	1.185	0.47	-	-	-	-	
Maltose	5	0.56 (0.00)	0.58	1.041	0.42	0.58 (0.02)	0.59	1.041	0.42	$a_{Ca^{2+}} = 0.0005 \cdot s + 0.00055$ (SMUF)
	10	0.59 (0.03)	0.60	1.086	0.43	0.60 (0.05)	0.61	1.076	0.44	$R^2 = 0.953$
	15	0.61 (0.03)	0.63	1.131	0.45	0.62 (0.03)	0.63	1.111	0.44	$a_{Ca^{2+}} = 0.0004 \cdot s + 0.00057$ (skim milk)
	20	0.66 (0.03)	0.65	1.176	0.47	0.65 (0.02)	0.65	1.146	0.46	$R^2 = 0.974$
Trehalose	5	0.58 (0.02)	0.59	1.059	0.42	-	-	-	-	
	10	0.62 (0.01)	0.62	1.122	0.45	-	-	-	-	$a_{Ca^{2+}} = 0.0005 \cdot s + 0.00055$ (SMUF)
	15	0.66 (0.01)	0.66	1.185	0.47	-	-	-	-	$R^2 = 0.987$

[a] All the values of Ca^{2+} activities and standard deviations were obtained by using their original values multiplying by a factor of 1000. The experiment was conducted in triplicate.
[b] The Ca^{2+} activities were measured by Ca-ISE method
[c] The Ca^{2+} activities were calculated by equations, which were obtained by linearly fitting the measured Ca^{2+} activities as a function of sugar concentrations (see the column Fit Equations).
[d] Ca^{2+} activity ratio between the calculated Ca^{2+} activity and the measured Ca^{2+} activity in absence of sugars.
[e] The adjusted Ca^{2+} activity coefficient, calculated by using equation (15)
[f] Based on 3 decimal Ca^{2+} activity data; s is concentration of sugar (%) (w/w)

5.4.2.2 Hydration effect

Figure 2a and 2d show that the concentrations of free Ca^{2+} and Mg^{2+} ions in SMUF do not change significantly throughout the range of sucrose concentrations, whereas in skim milk they appear to decrease as the sucrose concentration increases. It seems that ion pairs are formed in skim milk, but not in SMUF in the presence of sucrose. **Figure 2f** depicts that the conductivity has a linear relationship with the inverse of viscosity in SMUF, so there is no evidence for ion pairs formation in this case. However, this is less clear for milk. These results indicate that the effect of sucrose on ionic properties of skim milk differ from those for SMUF, which apparently can not be explained solely by excluded volume effects.

Sucrose can bind free water molecules and decrease water activity. This leads to a change in hydration properties of solutes. The differences in solute-water interactions and changes to the hydration sphere around solute species (Chuy & Bell, 2006) may account for the difference in ion pair formation between SMUF and skim milk. The major compositional differences between SMUF and skim milk are the proteins, namely casein micelles and whey proteins, and lactose. Studies suggest that the preferential exclusion of sugar molecules from the casein domain results in preferential hydration of the caseins (Considine & Flanagan, 2009; Mora-Gutierrez & Farrell H.M, 2000; Mora-Gutierrez et al., 1997) and of the globular proteins (Considine & Flanagan, 2009; Semenova et al., 2002). The increase in hydration of milk proteins, and the ability of sucrose to bind water, can cause considerable dehydration of ions, resulting in ion pair formation in milk. Although MSA theory does not directly consider the solute-water interaction, it is possible to integrate the hydration properties of ions into MSA model, for instance, by taking into account the dependence of the size of hydrated ions on sucrose

concentration. This would require experimentally determining the change in diameters of hydrated ions as an increase in sucrose concentration. Thus, the MSA model may possibly describe the changes in free ion concentrations in case of changes in hydration.

In addition, the ion equilibria between aqueous phase and colloidal phase are not significantly affected throughout the range of sucrose concentration as shown by the concentrations of ions in milk serum (**Table 7**). The influence of sucrose on ion speciation in milk appears to occur only in milk serum.

Table 7. Concentration (mmol/kg water) of major ions in milk serum [a]

		[Ca] (SD) [b]	[K] (SD)	[Mg] (SD)	[Na] (SD)	[P][c] (SD)
Sugars	% (w/w)	(mmol/kg water)				
None	0	11.1 (0.2)	45.8 (1.1)	3.9 (0.1)	20.7 (0.3)	15.8 (0.5)
Sucrose	10	10.7 (0.7)	46.4 (1.0)	3.8 (0.3)	20.4 (0.8)	15.9 (0.2)
	20	10.9 (0.1)	45.4 (1.0)	3.7 (0.1)	19.9 (0.4)	15.4 (1.2)
	30	11.1 (0.4)	44.3 (1.4)	3.6 (0.1)	19.9 (0.8)	16.0 (0.6)
Lactose	10	11.4 (0.2)	48.8 (0.9)	3.9 (0.1)	21.4 (0.4)	16.6 (0.2)
Maltose	10	11.5 (0.3)	48.8 (0.4)	4.0 (0.2)	21.5 (0.2)	16.2 (0.4)

[a] Concentrations (mmol/kg water) of the major ions in skim milk: [Ca] = 36.4, [Mg] = 5.8, [Na] = 22.1, [K] = 50.2, [P_i] = 22.6, [Cl] = 34.0, [Citrate] = 11.2, [SO_4] = 1.1;

[b] n=2

[c] [P] represents the sum of the concentration of inorganic phosphate and organic phosphate in milk serum, which is measured by ICP-AES.

5.4.3 Comparison among sugars

With respect to the four disaccharides, sucrose and trehalose are

non-reducing sugars, while lactose and maltose are reducing sugars. **Table 6-8** summarize the influence of the four disaccharides on the thermodynamic properties of SMUF and skim milk. Generally, all sugars give rise to similar effects on pH, Ca^{2+} activity and concentration, Ca^{2+} activity coefficient, conductivity, water activity and ion equilibria. This suggests that it is the excluded volume effect, which is the main factor in the impact on thermodynamic properties of ions in the solution, rather than the type of sugars. Still, slight differences in solution properties remain. First, the reducing sugars resulted in slightly lower pH than the non-reducing sugars in SMUF, which is possibly due to a release of hydrogen ions from the active hydroxyl group at their reducing end. Second, trehalose causes slightly lower water activities and higher Ca^{2+} activities than did the other sugars in SMUF. This may be due to its binding a larger number of water molecules than do maltose and sucrose, thus affecting the structure of water to a greater extent (Lerbret, Bordat, Affouard, Descamps & Migliardo, 2005).

Table 8. Comparison of the influence of disaccharides on electrolyte properties of SMUF and skim milk

		Ca^{2+} concentration (mmol/kg) (SD)[b]		pH (SD)[a]		Conductivity (mS/cm) (SD)[a]		a_w (SD)[a]	
Sugars	% (w/w)	SMUF	Skim Milk	SMUF	Skim Milk	SMUF	Skim Milk	SMUF	Skim Milk
None	0	1.73 (0.08)	2.16 (0.23)	6.68 (0.02)	6.66 (0.02)	6.74 (0.04)	5.96 (0.09)	0.999 (0.001)	0.996 (0.001)
Sucrose	5	-	-	6.65 (0.04)	6.63 (0.01)	5.85 (0.07)	5.09 (0.05)	0.997 (0.001)	0.994 (0.001)
	10	1.77 (0.06)	2.10 (0.16)	6.60 (0.04)	6.61 (0.01)	5.05 (0.05)	4.37 (0.10)	0.995 (0.001)	0.990 (0.001)
	20	1.74 (0.04)	1.91 (0.04)	6.50 (0.04)	6.58 (0.01)	3.58 (0.04)	2.59 (0.08)	0.987 (0.001)	0.982 (0.001)
	30	1.74 (0.08)	1.86 (0.09)	6.43 (0.04)	6.54 (0.01)	2.32 (0.03)	1.92 (0.06)	0.977 (0.001)	0.970 (0.001)
	40	-	-	6.36 (0.05)	6.52 (0.02)	1.34 (0.02)	1.02 (0.03)	0.962 (0.001)	0.952 (0.001)
Lactose	5	-	-	6.61 (0.01)	6.63 (0.01)	5.83 (0.04)	5.07 (0.06)	0.997 (0.001)	0.994 (0.001)
	10	1.80 (0.03)	2.21 (0.12)	6.55 (0.04)	6.61 (0.01)	4.98 (0.04)	4.33 (0.08)	0.994 (0.001)	0.992 (0.001)
	15	-	-	6.49 (0.02)	-	4.19 (0.04)	-	0.993 (0.001)	-
Maltose	5	-	-	6.60 (0.04)	6.64 (0.01)	5.81 (0.06)	5.07 (0.06)	0.997 (0.001)	0.994 (0.001)
	10	1.73 (0.05)	2.15 (0.10)	6.55 (0.05)	6.61 (0.01)	4.99 (0.06)	4.35 (0.07)	0.995 (0.001)	0.992 (0.001)
	15	-	-	6.50 (0.02)	6.59 (0.01)	4.21 (0.05)	3.59 (0.06)	0.992 (0.001)	0.987 (0.001)
	20	-	-	6.49 (0.02)	6.56 (0.03)	3.49 (0.05)	2.54 (0.06)	0.986 (0.001)	0.984 (0.001)
Trehalose	5	-	-	6.62 (0.01)	-	5.81 (0.01)	-	0.996 (0.001)	-
	10	1.84 (0.03)	-	6.58 (0.01)	-	4.94 (0.01)	-	0.992 (0.001)	-
	15	-	-	6.53 (0.01)	-	4.12 (0.01)	-	0.990 (0.001)	-

[a] n=3

[b] n=2

5.5 Conclusion

In conclusion, the MSA theory appears to satisfactorily explain the influence of disaccharides on the thermodynamic properties of ions in $CaCl_2$-KCl-K_3Citrate solution, and to qualitatively describe ion properties in SMUF and skim milk upon addition of disaccharides. The large increase in ion activity coefficient, which is caused by excluded volume effects, accounts for the significant increase in Ca^{2+} activity and decrease in pH in milk systems. However, the excluded volume effect is not the only explanation for the decrease in free Ca^{2+} and Mg^{2+} concentration in skim milk enriched with sucrose. The finding that sucrose addition causes preferential hydration of

milk proteins may provide insights to understand the decrease in free Ca^{2+} and Mg^{2+} concentrations in milk. All the disaccharides do not appear to significantly alter the original ion equilibria between serum and colloidal phase. The influence of disaccharides on the free ion concentrations seems to happen only in milk serum. Last, but not least, no significant difference is found among sugars since all the sugars have similar impacts on the thermodynamic properties of ions in milk systems

Further details of the calculation in MathCAD of Ca^{2+} and H^{+} ion activity coefficients in the $CaCl_2$-KCl-K_3Citrate-Sucrose solutions can be obtained from the authors upon request.

Acknowledgement

This work is funded by FrieslandCampina Innovation Europe. The authors are grateful to Arie vanden Berg and Peter Nobels from the Department of Soil Quality, Wageningen University, for performing ICP-AES measurement. We would also like to thank MSc student Meilianti for performing part of experimental work.

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Chapter 6

Quantification and calculation of the ion composition in reconstituted skim milk and the consequences of adding electrolytes: HCl, NaCl, KCl, $CaCl_2$ and Na_2HPO_4

This Chapter has been submitted as:
Gao, R., van Leeuwen, H. P., van Valenberg, H. J. F., Eisner, M. D., & van Boekel, M. A. J. S. Quantification and calculation of the ion composition in reconstituted skim milk and the consequences of adding electrolytes: HCl, NaCl, KCl, $CaCl_2$, and Na_2HPO_4.

Abstract

This study presents a model to calculate the ion partitioning in milk and milk to which electrolytes are added. The model, named milk ion speciation (MIS) model, is an extension of the earlier published dynamic ion speciation (DIS) model which describes the ion equilibria in the aqueous phase of milk. The micellar phase, which contains colloidal calcium phosphate (a mixture of acidic and basic calcium phosphate and magnesium phosphate) and casein bound cations, is included in the MIS model. Experiments were carried out with milk and milk to which HCl, NaCl, KCl, $CaCl_2$ and Na_2HPO_4 were added. The modelled results were in general agreement with experimental results and literature data for all conditions. Thus, the MIS model appears to be able to predict and explain the behaviour of ions in milk systems. This model can be further implemented in dairy industry as a useful tool for new product development.

Keywords**:** Ion equilibria, colloidal calcium phosphate, milk, pH, NaCl, KCl, $CaCl_2$, phosphate, ion speciation model

6.1 Introduction

The milk mineral fraction is a minor fraction in terms of milk composition, but plays a crucial role in the conformation and stability of casein micelles (Fox & McSweeney, 1998; Holt, 1997; Walstra & Jenness, 1984). Milk salts are distributed between the aqueous phase and colloidal phase. The ions in the aqueous phase are dissolved in the form of either free ion such as Ca^{2+} or ion complexes such as $CaCitrate^{-}$. The ions in the colloidal phase are mainly in the form of solid calcium phosphates and cation-protein complexes. As a result, ion equilibria are slowly formed between the aqueous phase and colloidal phase. Alterations of the ion equilibria induce significant changes in the ionic compositions between the colloidal phase and aqueous phase. This has effects on the physico-chemical properties of casein micelles and further on the stability of products during processing and storage (De La Fuente, 1998; Fox & McSweeney, 1998; Huppertz & Fox, 2006). Therefore, it is of great practical importance to develop a suitable model to describe ion equilibria in milk.

Models that describe the ion equilibria of milk-like systems have been developed since the 1980s. Wood et al. (1981) attempted to calculate the ion concentrations in a simulated milk salt solution. For the ease of calculation, assumptions were made in the model such as no presence of magnesium, phosphate esters and proteins, and potassium ions equivalent to sodium ions. Lyster (1981) extended the model from Wood et al. (1981), and provided the possibility to calculate a solid phase in equilibrium at different temperature and pH values. Holt et al. (1981) developed a model to describe the ionic composition in milk diffusate, including all the relevant components in milk diffusate except milk proteins, and the calculated free Ca^{2+} and Mg^{2+}

concentrations were in reasonable agreement with experiment. These models have been used for many years as useful tools to describe ionic compositions in the aqueous phase of milk or dairy products (Gaucheron et al., 2000; Kent et al., 1998; Le Graët & Gaucheron, 1999; Morris et al., 1988; Philippe et al., 2003), in order to understand the interrelationship of the major ions with physico-chemical properties of casein micelles. However, these models did not include the colloidal calcium phosphate (CCP) and casein bound cations in casein micelles. Thus, their application in the prediction of ion partitioning in milk and dairy products is still limited. More recently, Holt (2004) developed an equilibrium thermodynamic model for calculation of the ion partitioning in milk in which the colloidal calcium phosphate is assumed to be present in the form of calcium phosphate nanoclusters. A generalized empirical formula for the calcium phosphate nanoclusters was used to define the molar ratios of small ions (Ca, Mg, P_i and citrate) to a casein phosphorylated sequence (Holt, 2004). This model provided a reasonable prediction of ion partitioning in milk in comparison with the experimental results of White and Davies (1958, 1963). However, this model requires further validation with more ion compositional data of milk samples at different conditions. Mekmene et al. (2009) presented a model to predict the ion partitioning between the micellar phase and aqueous phase in milk and mineral-enriched milks such as addition of NaCl, $CaCl_2$, Na_3Citrate and Na_2HPO_4. This latter model is based on the earlier model of Holt et al. (1981) by integrating interactions of cations in casein micelles and taking into account the solubility of calcium phosphate in the micellar phase. The model was validated to give predictions that generally agreed with literature experimental results, though differences in the order of 10 to 20 % still remain between model and experimental results. Moreover, this model is used to predict ion partitioning in milk at constant pH value of 6.75. However,

in reality, addition of electrolytes to milk induces changes in pH. Very recently, Mekmene et al. (2010) presented a model to simulate the ion equilibria of milk upon acidification. The model gave reasonable predictions of ion equilibria compared to experimental results. However, their model is not a generic model to describe the ion equilibria of milk at various conditions. Moreover, magnesium is considered to exist in the micellar phase as $Mg_3citrate_2$, which is an incorrect assumption since Mg is not present in the form of $Mg_3citrate_2$ as reported by Alexander and Ford (1957). Consequently, a generalized model to describe the ion equilibria of milk under various conditions should be developed.

The aim of our work is to present a model to accurately predict ion partitioning in milk and milk samples with addition of electrolytes. For this purpose, the previous model of Gao et al. (2010), which describes the ion speciation in simulated milk ultrafiltrate (SMUF) at equilibrium as well as at non-equilibrium state, will be extended to build a model for milk. All the major components of aqueous phase are included, and the colloidal calcium phosphate and interactions between cations and casein proteins are integrated in this model. To validate the model, experiments will be carried out in milk at different conditions such as pH, the addition of NaCl, KCl, $CaCl_2$, and Na_2HPO_4. The results of the model calculation are compared with experimental results and also with literature data.

6.2 Materials and Methods

6.2.1 Description of the model

The computer program AESolve (Halotec Instruments, The Netherlands) was used as the basis to calculate the ion speciation in milk at various conditions.

The model is named milk ion speciation (MIS) model and is an extension of the DIS model described by Gao et al. (2010), which is used to calculate the ion speciation in simulated milk ultrafiltrate (SMUF) under different conditions. The MIS model includes all the components of SMUF, phosphate ester fraction (regarded as glucosyl-1-phosphate^{2-}) and organic acid (regarded as lactate^{-}) representing the serum phase, and colloidal calcium phosphate (CCP), and casein phosphoserine residues (CN-SerP^{2-}) as the micellar phase. The CCP is considered as a group of undissolved and amorphous calcium phosphate precipitates. However, components such as lactose, carboxyl groups of glutamic and aspartic acids on caseins, casein micelles and whey proteins were left out of the MIS model. In addition, micellar citrate is not included in the micellar phase of the MIS model. One reason is that its exact location in the micellar phase is not yet established (Holt, 2004). Another reason is that inclusion of micellar citrate leads to exceeding computation capacity of the program. Rather, the focus of the MIS model was on calculation of the ion partitioning between the salts dissolved in the serum phase and CCP and cations bound to CN-SerP^{2-} residues in the micellar phase. All the relevant association constants (K_{ass}) and solubility products (K_{sp}) employed in the MIS model are listed in **Table 1** and **2**, respectively.

The nature of colloidal calcium phosphate in milk, which is mainly determined based on the molar ratio (Ca/P_i) between the non-diffusible calcium and micellar inorganic phosphate (Mekmene et al., 2009), has been the subject of debate over many years. Some studies have suggested that CCP is a more acidic phase, such as some brushite-type structure ($CaHPO_4 \cdot 2H_2O$) (Chaplin, 1984; Holt, 1985). Other studies reported the Ca/P_i ratio about 2 by McGann et al. (1983), and about 1.75-1.84 by Dalgleish and Law (1989). Moreover, Lucey and Horne (2009) suggested that the form of CCP is a basic

form based on various titration studies, the real pK_a values for phosphate in milk and the distinctive acid-base properties of milk. Our experimental result of Ca/P_i molar ratio gave a mean value of 1.84 ± 0.10. Thus, we consider CCP to be of a more basic nature consisting of a mixture of acidic and basic calcium phosphate as well as trimagnesium phosphate. At pH near 6.70 different calcium phosphate phases may actually coexist as it is a pH of phase transition for acidic and basic calcium phosphates (Van Kemenade & De Bruyn, 1987). Since the experimental Ca/P_i ratio does not correspond to any known calcium phosphate phase, it must be a mixture of calcium phosphate phases with Ca/P ratio above and below 1.84. In the MIS model, the CCP is considered to contain $CaHPO_4{\cdot}2H_2O$ and $Ca_4(PO_4)_2(OH)_2$ as well as $Mg_3(PO_4)_2$ as shown in **Table 2**. The $CaHPO_4{\cdot}2H_2O$ is the likely acidic form of calcium phosphate according to Chaplin (1984) and Holt (1985). The basic form $Ca_4(PO_4)_2(OH)_2$, which appears similar to hydroxylapatite, is an assumed calcium phosphate phase.

For calculation of ion speciation, the ionic composition of milk is required as the input data. In the MIS model, the total concentrations (mmol/kg water) of the major components are used: [Ca] (32.1), [Mg] (5.6), [K] (47.4), [Na] (21.0), [Cl] (37.4), [P_i] (23.0), [Glc-1-P] (3.7), [CN-SerP] (3.7), [Citrate] (10.9), [SO_4] (1.1), [Lactate] (2.0), which are obtained from our experimental measurements as shown in **Table 3**. The mass balance equation for each component is constructed and consists of a sum of the concentrations of various forms. The ensuing equations are nonlinear algebraic equations, which can be solved numerically to obtain activities and concentrations for all the ions and ion complexes. Other properties such as the ionic strength, pH, ion activity coefficients and water activity can also be calculated.

Table 1 Logarithm of association constants (pK_{ass}) of ions and ion complexes used in the MIS model

	H^+	Ca^{2+}	Mg^{2+}	K^+	Na^+
$Citrate^{3-}$	6.4[a]	5.15[a]	5.15[a]	1.20[a]	1.30[a]
$HCitrate^{2-}$	4.76[a]	3.3[c]	2.7[a]	1[a]	1[a]
$H_2Citrate^-$	3.13[a]	1.45[a]	1.18[a]	0[a]	0[a]
PO_4^{3-}	12.67[b]	6.46[a]	4.92[a]	1.7[e]	2[a]
HPO_4^{2-}	7.22[b]	3.0[a]	2.92[a]	1.4[e]	1.7[e]
$H_2PO_4^-$	2.17[b]	1.04[a]	1.08[a]	0[a]	0[a]
Cl^-	-	0.8[d]	1.1[e]	0[a]	0[a]
HCO_3^-	6.35[a]	1.26[b]	1.28[a]	0[a]	0[a]
SO_4^{2-}	-	2.31[a]	2.23[a]	0.85[a]	0.7[a]
Glc-1-PH^-	-	-	-	-	-
Glc-1-P^{2-}	6.50[a]	2.50[a]	2.47[a]	0.78[a]	0.85[a]
$Lactate^-$	3.86[a]	1[a]	1[a]	0.7[a]	0.7[a]
Casein- $SerPH^-$	2.30[e]	-	-	-	-
Casein-$SerP^{2-}$	6.40[a]	4.75[e]	4.5[e]	-	-

[a] Holt et al. (1981)

[b] Smith and Martell (1981)

[c] Martell and Smith (1979)

[d] Davies (1962)

[e] Estimated

Table 2 Solubility products (K_{sp}) of colloidal calcium phosphate (CCP) and other likely formed solid precipitates in the MIS model.

Solid precipitates	Chemical formula	Solubility Product K_{sp}
Colloidal calcium phosphate:		
Calcium phosphate (Acidic type)	$CaHPO_4 \cdot 2H_2O$	$6.76 \cdot 10^{-7}$ [d]
Calcium phosphate (Basic type)	$Ca_4(PO_4)_2(OH)_2$	$7.24 \cdot 10^{-46}$ [d]
Trimagnesium phosphate	$Mg_3(PO_4)_2$	$1.74 \cdot 10^{-29}$ [d]
Other solid precipitates:		
Amorphous tricalcium phosphate	$Ca_3(PO_4)_2 \cdot xH_2O$	$6.17 \cdot 10^{-28}$ [d]
Octacalcium phosphate	$Ca_4(HPO_4)_3 \cdot 5H_2O$	$1.26 \cdot 10^{-49}$ [b]
Tricalcium citrate tetrahydrate	$Ca_3Citrate_2 \cdot 4H_2O$	$5.01 \cdot 10^{-18}$ [a]
Magnesium phosphate	$MgHPO_4$	$1.51 \cdot 10^{-6}$ [c]

[a] Walstra & Jenness (1984)

[b] Johnsson & Nancollas (1992)

[c] Taylor et al. (1963)

[d] Estimated

6.2.2 Experimental Set-up

All experiments were carried out at 20 ± 1.0 ºC and all glassware was washed in phosphate-free detergent, soaked in 1.4 M nitric acid and rinsed in deionized water (Millipore, Amsterdam, The Netherlands). All chemicals used were purchased from Fluka, with 99.5 % purity. Skim milk was reconstituted by dissolving 1 portion of low heat skim milk powder (Nilac, NIZO, the Netherlands) in 9 portions of deionized water, as described by Gao et al. (2009). The skim milk samples with added electrolytes were stored and equilibrated under continuous stirring for 24 hours before measurement. 0.02 % (w/w) sodium azide was added to each milk sample to prevent microbial growth.

6.2.2.1 Addition of hydrogen chloride (HCl)

Skim milk samples were acidified with 6 M HCl from pH 6.7 to 4.5. The experiment was repeated in triplicate.

6.2.2.2 Addition of sodium chloride (NaCl) or potassium chloride (KCl)

NaCl or KCl as solid was added to skim milk samples in the range between 0 and 0.3 mol/kg water. The experiment was carried out in triplicate.

6.2.2.3 Addition of calcium chloride ($CaCl_2$)

Skim milk samples were enriched with 5 M $CaCl_2$ solution up to a concentration of 0.01 mol/kg water. All data were collected in triplicate.

6.2.2.4 Addition of disodium phosphate ($Na_2HPO_4 \cdot 2H_2O$)

Disodium phosphate (DSP) as solid was added to skim milk samples. The concentration of DSP in milk samples was in the range 0 – 0.6 % (w/w). The experiment was conducted in triplicate.

6.2.3 Methods

6.2.3.1 Total concentration of major ions

Total concentrations of Ca, Mg, K, Na, and P in skim milk were measured by first diluting skim milk samples 100 times and then diluting 10 times with 1.4 M HNO_3 (final concentration 0.14 M HNO_3). The diluted samples were analyzed by inductively coupled plasma-Atomic emission Spectrometry (ICP-AES) (Varian Vista-Pro radial system, Mulgrave, Australia). For the concentrations of anions in skim milk, the skim milk samples were diluted with deionized water 1000 times. The total concentration of chloride, sulphate, citrate, and phosphate were determined by anion exchange

chromatography according to the method described by Gaucheron et al. (1996).

Milk serum was obtained by ultracentrifugation of skim milk samples (100,000 *g*, 20 °C for 1 hour) in a Beckman L-60 ultracentrifuge with a 70 Ti rotor (Beckman Instruments Inc, Germany). The total concentrations of ions in milk serum were determined by ICP-AES and anion exchange chromatography. A similar procedure of dilution was used for milk serum as described for milk.

6.2.3.2 Determination of free Ca^{2+} and Mg^{2+} ions

The concentrations of free Ca^{2+} and Mg^{2+} ions in skim milk samples were determined by the Donnan Membrane Technique as described by Gao et al. (2009). Free Ca^{2+} ion activity was determined by a calcium ion selective electrode (Orion 9720, Thermo, Beverly, USA) as described by Gao et al. (2010).

6.2.3.3 pH measurement

The pH of skim milk samples was measured by a pH electrode (Orion 8172BNWP, Thermo, Beverly, USA). The pH electrode was first calibrated in standard buffer solutions pH 4.00 and pH 7.00. Subsequently, the pH electrode was immersed in the samples and a stable reading was obtained after approximately 3 minutes.

6.3 Results and Discussion

6.3.1 Ion partitioning between aqueous and micellar phase at pH 6.7

Table 3 shows the experimental results of the distribution of the major ions

between milk serum and micellar phase at pH 6.7. The results are in general agreement with literature data (Gaucheron, 2005; Holt, 1985; Walstra & Jenness, 1984). Sodium, potassium, chloride and sulphate ions are present only in the serum phase, while nearly 1/3 calcium, 2/3 magnesium, 1/2 inorganic phosphate and 95 % citrate ions are present in the serum phase, and the rest exists in the micellar phase as colloidal calcium phosphate or bound to casein phosphoserine residues. Only a small fraction of Ca and Mg remains free. The concentrations of free Ca^{2+} and Mg^{2+} ions are 2.05 and 0.68 mmol/kg water, respectively (**Table 3**), again in line with literature results (Gao et al., 2009; Geerts et al., 1983; Holt, 1985) and modelled results (Holt et al., 1981).

Table 3. Minerals distribution between milk serum and micellar phase at pH 6.7 [a, b, c].

Constituents	Concentration (mmol/kg water)	Constituents	Concentration (mmol/kg water)
Calcium Total	32.1 (2.2)	**Phosphate Total**	30.4 (1.9)
Micellar Ca	21.5	Casein seryl P	3.7
Serum Ca	10.6 (0.6)	Micellar P_i	11.7 (0.8)
Free Ca^{2+}	2.05 (0.16)	Serum P_i	11.3 (0.1)
% soluble Ca	33 %	Serum ester P	3.7
		% soluble P_i	49 %
Magnesium Total	5.6 (0.5)		
Micellar Mg	1.8	**Citrate Total**	11.4 (0.3)
Serum Mg	3.8 (0.2)	Serum Citrate	10.9 (0.1)
Free Mg^{2+}	0.68 (0.05)	Micellar Citrate	0.5
% soluble Mg	68 %	% soluble citrate	95 %
Sodium Total	21.0 (1.0)	**Chloride Total**	37.4 (2.1)
Free Na^+	18.8 (0.5)		
		Sulphate Total	1.1 (0.1)
Potassium Total	47.4 (3.5)		
Free K^+	45.9 (1.2)	**Organic acid Total**	2.0

[a] ( ) represents standard deviation (n = 10)

[b] Serum ester P is considered to be glucosyl-1-phosphate

[c] Organic acid is considered to be lactic acid, which is present as lactate at pH 6.70; the concentration is estimated.

Table 4 shows the calculated ion partitioning between the serum phase and micellar phase using the MIS model. The results are in line with the experimental results except for inorganic phosphate shown in **Table 3**. The MIS model predicts an amount of inorganic phosphate in micellar phase (13.4 mmol/kg water) slightly higher than the experimental data (11.7

mmol/kg water). The solubility products of calcium phosphate phases used in the model may account for more phosphate ions to form CCP. Meanwhile, without the presence of citrate ions in the micellar phase, calcium and magnesium ions complex more phosphate ions to form CCP. Besides the calculated ion distribution, the ion composition in milk serum was also calculated by the MIS model, as shown in **Table 5**. The Ca^{2+} and Mg^{2+} ions were mainly complexed by $citrate^{3-}$ and to a lesser extent by HPO_4^{2-}, while the Na^+ and K^+ ions were mainly present in the free form. The calculated concentrations of free Ca^{2+}, Mg^{2+}, Na^+ and K^+ ions were comparable to the experimental results reported in **Table 3**. Moreover, the calculated concentrations of ions and ion complexes are generally in accordance with the modelled data by Holt et al. (1981) and Mekmene et al. (2009). However, the concentrations of some ion species, such as $CaCitrate^-$, Cl^-, HPO_4^{2-}, K^+ and $KHPO_4^-$ are different from the data of Holt et al. (1981). This could be due to the fact that the total concentrations of these ions used in the MIS model are different from those reported by Holt et al (1981). In addition, the calculated ionic strength of milk serum had a value of 0.08 mol/kg water, which was a bit higher than the reported value of 0.073 mol/kg water (Holt et al., 1981). The higher concentrations of potassium and chloride ions in the MIS model may account for the difference.

Table 4. MIS model calculation of salts partition (mmol/kg H_2O) between serum phase and micellar phase.

	Total	Serum	Micellar phase
Ca	32.1	10.6	21.5
Mg	5.6	3.75	1.85
Na	21.0	21.0	0.0
K	47.4	47.4	0.0
P_i	23.0	9.6	13.4
Glucosyl-1-P	3.7	3.7	0.0
Citrate	10.9	10.9	0.0
Cl	38.4	37.4	0.0
SO_4	1.1	1.1	0.0
Lactate	2.0	2.0	0.0
Casein seryl P	3.7	0.0	3.7

Table 5 Calculated concentrations (mmol/kg water) of ions and complexes in milk serum by the MIS model [a, b, c] versus literature values from Holt et al. (1981)

		Cation complex			
Anion	Free ion	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
$Citrate^{3-}$	0.50 (0.26)	7.51 (6.96)	2.67 (2.02)	0.05 (0.04)	0.03(0.03)
$HCitrate^{2-}$	0.08 (0.04)	0.04 (0.01)	- (-)	0.01 (-)	0.01 (-)
$H_2Citrate^{-}$	- (-)	- (-)	- (-)	- (-)	- (-)
PO_4^{3-}	- (-)	- (-)	- (-)	- (-)	- (-)
HPO_4^{2-}	2.45 (2.65)	0.67 (0.59)	0.20 (0.34)	1.05 (0.52)	1.04 (0.39)
$H_2PO_4^{-}$	4.00 (7.50)	0.03 (0.07)	0.01 (0.04)	0.11 (0.18)	0.05 (0.10)
$Glc\text{-}1\text{-}PH^{-}$	1.02 (0.50)	- (-)	- (-)	- (0.01)	- (0.01)
$Glc\text{-}1\text{-}P^{2-}$	2.10 (1.59)	0.18 (0.17)	0.06 (0.07)	0.22 (0.14)	0.13 (0.10)
$Lactate^{-}$	1.93 (2.98)	0.01 (0.03)	- (0.02)	0.04 (0.04)	0.02 (0.02)
Cl^{-}	36.8 (30.9)	0.17 (0.26)	0.12 (0.07)	1.00 (0.68)	0.50 (0.39)
HCO_3^{-}	0.04 (0.32)	- (-)	- (-)	- (-)	- (-)
SO_4^{2-}	0.89 (0.96)	0.05 (0.07)	0.02 (0.03)	0.11 (0.10)	0.04 (0.04)
Free ion	-	1.91 (2.00)	0.68 (0.81)	44.9 (36.3)	19.2 (20.9)

[a] Concentrations (mmol/kg water) of the major ions in milk serum: [Ca] = 10.6, [Mg] = 3.8, [Na] = 21.0, [K] = 47.4 $[P_i]$ = 9.6, [Cl] = 38.4, [Citrate] = 10.9, $[SO_4]$ = 1.1; ionic strength of milk serum was 0.08 mol/kg water (calculated); pH of SMUF was 6.70.

[b] Values shown as ( ) were calculated concentrations (mmol/L) of ion species in a typical milk diffusate by Holt et al. (1981), in which [Ca] = 10.2, [Mg] = 3.4, [Na] = 22.0, [K] = 38.0, $[P_i]$ = 12.4, [Cl] = 32.3, [Citrate] = 9.4, $[SO_4]$ = 1.2; ionic strength was 0.073 M and pH was 6.70.

[c] Concentration shown as: - < 0.01 mmol/kg water

6.3.2 Addition of HCl

Skim milk was acidified by adding HCl in the pH range between 4.5 and 6.7. The measured concentrations of free Ca^{2+} and Mg^{2+} ions were found to increase up to 19.3 mmol/kg water and 3.1 mmol/kg water, respectively at pH 4.5 (**Figure 1**). Similarly, the Ca^{2+} ion activity increased over 10 fold (from 5.7E-04 to 6.2E-03) as the pH decreased from 6.7 to 4.5. The model calculation suggests that the addition of HCl causes protonation of anions such as $citrate^{3-}$ and HPO_4^{2-} in serum phase. This leads to remarkable dissociation of ion complexes such as $CaCitrate^-$, $CaHPO_4$ and $MgCitrate^-$ to release free Ca^{2+} and Mg^{2+} ions. The other reason is the solubilization of the CCP and the release of the bound Ca and Mg by phosphoserine residues into the serum phase as the pH drops. The ionic strength was therefore calculated to increase from 83 mmol/kg water to 137 mmol/kg water (**Table 6**). The calculated concentrations and activities of free Ca^{2+} and Mg^{2+} ions are generally in accordance with the experimental data, although the calculated Mg^{2+} concentrations are a bit higher (around 10%) than the experimental results (**Figure 1**). This is probably due to some uncertainty in the K_{sp} value of the solid trimagnesium phosphate ($Mg_3(PO_4)_2$). The model calculation suggests that trimagnesium phosphate completely solubilizes at pH above 6.3, resulting in a higher free Mg^{2+} concentration than experimental results. Changing the K_{sp} values can improve the fit of modelled data to experimental results, but will reduce the fit of the modelled Ca^{2+} concentrations and activities. Due to the great importance of calcium ions, the model was primarily optimized to match with the measured calcium activities and concentrations.

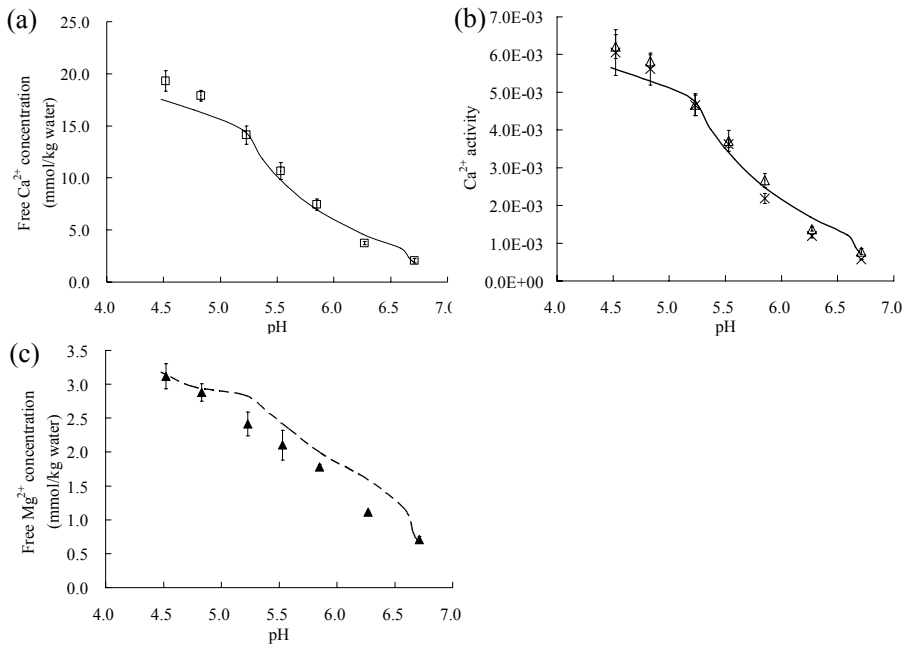

Figure 1. Effect of pH on ion composition of milk. (a) Free Ca^{2+} concentration as a function of pH: (□) Ca^{2+} concentration measured by DMT; (—) Model calculation. (b) change of Ca^{2+} activity with pH: (△) DMT (activity coefficients used from the model calculation); (×) Ca-ISE; (**—**) Model calculation. (c) Free Mg^{2+} concentration as a function of pH: (▲) DMT; (– –) Model calculation.

Table 6. The predicted ionic strengths (*I*) (mmol/kg H_2O) of milk enriched with different electrolytes.

pH (addition of HCl)	*I*	Addition of NaCl or KCl (mmol/kg H_2O)	*I*	Addition of $CaCl_2$ (mmol/kg H_2O)	*I*	Addition of $Na_2HPO_4{\cdot}2H_2O$ (%) w/w	*I*
6.7	80	0	80	0	80	0.0%	80
6.27	92	50	131	1	81	0.1%	92
5.85	100	100	177	2	84	0.2%	102
5.53	108	150	222	4	86	0.3%	112
5.23	126	200	265	5	87	0.4%	123
4.83	133	250	307	7.5	91	0.5%	133
4.52	137	300	348	10	95	0.6%	143

Figure 2 shows the calculated distribution of some major ions in the pH range between 4.5 and 8.0. The model calculation suggests that four zones can be identified to describe the Ca, Mg and P_i ions distribution throughout the pH range. In zone I (pH 8.0 – 6.77), the model indicates that acidification resulted in a decrease in micellar Ca, Mg, and P_i and an increase in serum Ca, Mg and P_i. This can be explained by the fact that acidification leads to the solubilization of the CCP. In zone II (pH 6.77 – 6.59), surprisingly, the model suggests that micellar P_i increases, and serum P_i decreases as pH decreases, which shows an opposite effect compared to that in zone I. Micellar Ca and serum Ca behaved similarly as in zone I, but the change in concentrations of micellar Ca and serum Ca was sharper than that in Zone I. The model suggests that a phase transition occurs to different calcium phosphates in CCP in this narrow pH range. That is, the basic form calcium phosphate ($Ca_4(PO_4)_2(OH)_2$ is completely solubilized at pH 6.59; the acidic form calcium phosphate ($CaHPO_4{\cdot}2H_2O$) is formed at pH 6.77. It is interesting to see such a dramatic change of Ca and P_i partition in this narrow pH range. According to Van Kemenade and De Bruyn (1987), at a pH near 6.7, a phase

transition occurs for basic and acidic calcium phosphate phases. This indicates that our formulation of CCP in terms of calcium phosphate in the model may be correct to explain the partitioning of calcium and phosphate, but it still requires further experimental validation. Besides, the model suggests that serum Mg sharply increases since a large amount of trimagnesium phosphate is solubilized. However, literature data showed a less sharper increase of serum magnesium (Le Graët & Brule, 1993). A possible reason is, again, some uncertainty of the solubility product of trimagnesium phosphate. Another reason can be due to inaccurate formulation of micellar Mg in the micellar phase. According to Holt (1985), micellar Mg is partly bound directly to casein and partly associated with micellar calcium phosphate. However, little is known of the association between micellar Mg and micellar calcium phosphate at neutral pH of milk. Apparently, trimagnesium phosphate may not be the right form of micellar Mg in the micellar phase. More work on the form of micellar Mg in the model should be done. In zone III (pH 6.59 – 5.23), acidification leads to an enormous increase in serum Ca, Mg and P_i, and a significant decrease in micellar Ca, Mg and P_i. The model calculation suggested that the micellar Ca and P_i was completely solubilized at pH 5.23, which is in general agreement with literature data (Le Graët & Brule, 1993; Le Graët & Gaucheron, 1999). As the pH falls below 5.23 (zone IV), the serum P_i does not change any more. However, the protein-bound cations, especially Ca^{2+}, start to dissociate and are released to the serum phase. The model shows a slower increase in serum Ca as the pH decreases from 5.23 to 4.5. Meanwhile, there was still around 2 mmol/kg water protein-bound Ca at pH 4.5, which is comparable to literature results (Le Graët & Brule, 1993).

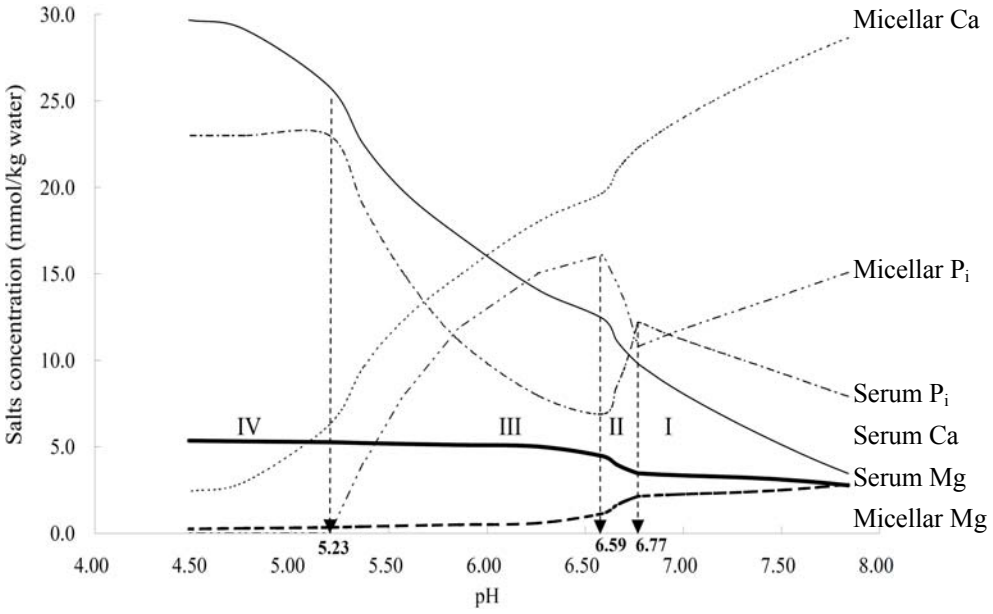

Figure 2. Model simulation of effect of pH on major ions partition between serum and micellar phase: (—) serum Ca; (---) micellar Ca; (– - –) serum inorganic phosphate (P_i); (– --) micellar inorganic phosphate (P_i); (▬) serum Mg; (– –) micellar Mg.

6.3.3 Addition of NaCl or KCl

Figure 3a and **3b** show an increase in concentrations of free Ca^{2+} and Mg^{2+} ions upon addition of NaCl or KCl throughout the concentration range. Specifically, the concentrations of free Ca^{2+} increased from 2.05 to 5.10 mmol/kg water with added NaCl and from 2.05 to 3.91 mmol/kg water with added KCl. The concentrations of free Mg^{2+} increased up to 1.22 and 1.08 mmol/kg water with added NaCl and KCl, respectively. Meanwhile, the model calculation was in accordance with the experimental results. The increase in Ca^{2+} and Mg^{2+} concentrations is, in the first place, due to the replacement of Ca^{2+} and Mg^{2+} ions from ion complexes in the serum phase

such as CaCitrate$^-$ and MgCitrate$^-$ by Na^+ or K^+ ions. Secondly, the addition of NaCl or KCl leads to solubilization of colloidal calcium phosphate due to an increase in ionic strength and also induces ion exchanges of Ca^{2+} and Mg^{2+} bound by casein phosphoserine residues with Na^+ or K^+. Thirdly, the addition of NaCl and KCl resulted in a decrease in pH with 0.13 and 0.09 units, respectively (**Figure 3d**), leading to slight solubilization of CCP. According to Gao et al. (2010), Na^+ or K^+ ions may form ion complexes with HPO_4^{2-}, causing the deprotonation of $H_2PO_4^-$. Moreover, Na^+ or K^+ may also displace the protons bound by milk proteins (Gaucheron et al., 2000; Huppertz & Fox, 2006). Figure 3c shows an increase in Ca^{2+} activity with addition of NaCl or KCl. Salting increases the ionic strength and decreases the Ca^{2+} ion activity coefficient. Apparently, the increase in concentration of Ca^{2+} outweighed the decrease in Ca^{2+} activity coefficient. The Ca^{2+} activity determined by DMT and Ca-ISE were generally agreeable in spite of some difference at lower ionic strength. The calculated Ca^{2+} activities matched with the experimental data within 10 % difference.

The addition of NaCl was observed to induce higher concentrations and activities of Ca^{2+} and Mg^{2+} ions, and lower pH than the addition of KCl (**Figure 3**). Similar phenomena were observed in simulated milk ultrafiltrate (SMUF) solution enriched with NaCl or KCl by Gao et al. (2010). According to the theory of the dependence of hydration strength on charge density (Collins, 1997; Collins et al., 2007), the affinity of Na^+ ions towards citrate^{3-} and HPO_4^{2-} is higher than that of K^+ ions. The same case applies to the affinity of Na^+ or K^+ with CN-SerP^{2-}. The difference between Na^+ and K^+ was also reflected in the model calculation, where different pK_{ass} values were employed (**Table 1**).

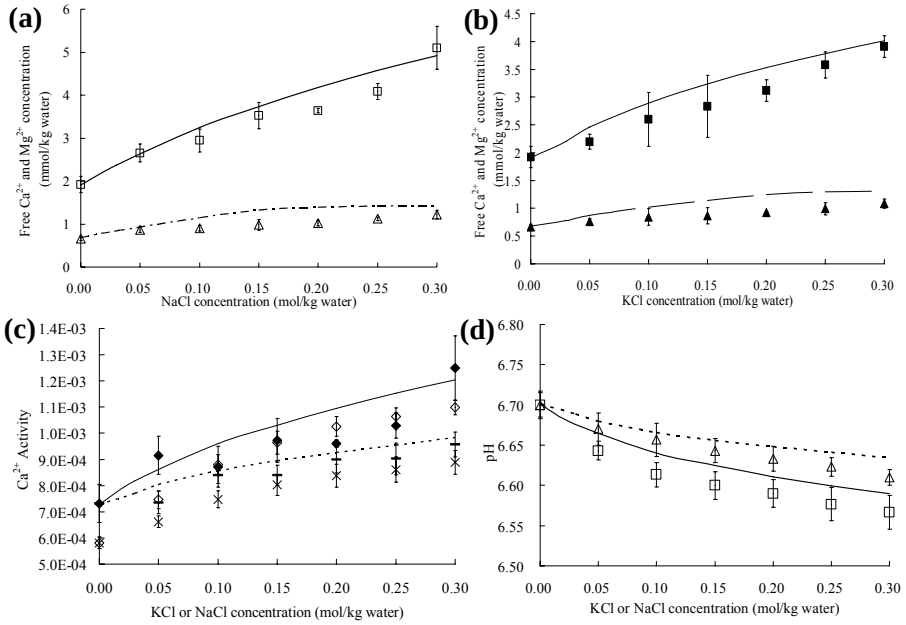

Figure 3. Effect of addition of NaCl or KCl on ion equilibria in milk. (a) NaCl Effect: (□) free Ca^{2+} concentration measured by DMT; (—) Calculated Ca^{2+} concentration; (△) free Mg^{2+} concentration measured by DMT; (– - –) Calculated Mg^{2+} concentration. (b) KCl Effect: (■) free Ca^{2+} concentration measured by DMT; (—) Calculated Ca^{2+} concentration; (▲) free Mg^{2+} concentration measured by DMT; (– –) Calculated Mg^{2+} concentration. (c) NaCl or KCl effect on Ca^{2+} activity: (◆) DMT method (NaCl); (◇) Ca-ISE method (NaCl); (—) Model calculation (NaCl); (-) DMT method (KCl); (×) Ca-ISE (KCl); (---) Model calculation (KCl). (d) pH as a function of added NaCl or KCl concentration: (□) measured (NaCl); (—) Model calculation (NaCl); (△) measured (KCl); (---) Model calculation (KCl).

Figure 4 shows the model calculation of ion partitioning after addition of NaCl and KCl. The ionic strength of milk was calculated to increase from 80 to 348 mmol/kg water with addition of 0 to 0.3 mol/kg water NaCl or KCl (**Table 6**). The addition of NaCl or KCl increased the concentrations of serum Ca, Mg and P_i and decreased the concentration of micellar Ca, Mg and P_i, which was in general agreement with literature data (Famelart et al., 1999; Mekmene et al., 2009). The increase in serum Ca and Mg is contributed partly by the solubilization of CCP and partly by the displacement of casein bound Ca^{2+} and Mg^{2+} by Na^+ or K^+ ions, since the increase in ionic strength leads to a decrease in activity coefficient and an increase in the stoichiometric solubility product of colloidal calcium phosphate and an increase in the dissociation of CN-SerPCa and CN-SerPMg. However, other studies (Gaucheron et al., 2000; Grufferty & Fox, 1985; Huppertz & Fox, 2006) reported that the addition of NaCl preferentially solubilized micellar Ca and Mg, but hardly influenced micellar P_i. The reason is that the aqueous phase is still saturated with calcium phosphate so that the increase in serum Ca restrains the solubilization of colloidal calcium phosphate (Gaucheron et al., 2000). Our model shows a reduction of $CaHPO_4{\cdot}2H_2O$ and an increase in $Ca_4(PO_4)_2(OH)_2$ of colloidal calcium phosphate, indicating that not all of the solubilized $CaHPO_4{\cdot}2H_2O$ goes to the serum phase. But the reduction of $CaHPO_4{\cdot}2H_2O$ is larger than the increase in $Ca_4(PO_4)_2(OH)_2$ in the micellar phase, resulting in a net increase in serum P_i.

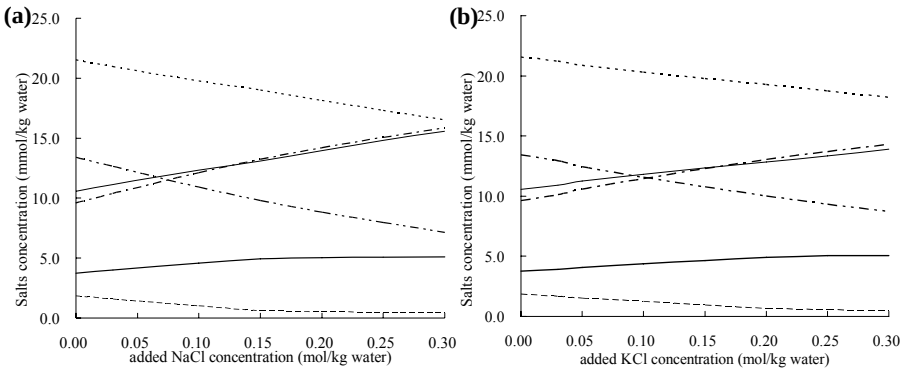

Figure 4. Model simulation of the effect of added NaCl or KCl on major ion partitioning between serum and micellar phase: (—) serum Ca; (---) micellar Ca; (– - –) serum inorganic phosphate (P_i); (– --) micellar inorganic phosphate (P_i); (▬) serum Mg; (– –) micellar Mg.

6.3.4 Addition of $CaCl_2$

Figure 5a shows that the concentrations of free Ca^{2+} and Mg^{2+} ions, respectively, increase up to 5.02 mmol/kg water and 1.23 mmol/kg water upon addition of $CaCl_2$ to a level of 10 mmol/kg water. The addition of $CaCl_2$ leads to an increase in the ionic strength from 80 to 95 mmol/kg water (**Table 6**), which induces a small decrease in the Ca^{2+} ion activity coefficient by approximately 0.03. The net result is that the Ca^{2+} activity increased up to 1.83E-03 (DMT results) or 1.68E-03 (Ca-ISE result) with the addition of 10 mmol/kg water $CaCl_2$ (**Figure 5b**). The changes in the concentrations and activities of free Ca^{2+} and Mg^{2+} ions were in accordance with the literature results (Tessier & Rose, 1958; Udabage et al., 2000). The model calculation is in agreement with the experimental results of the concentrations and activities of free Ca^{2+} and Mg^{2+} (**Figure 5a** and **5b**), and also gives similar predictions to the modelled results by Mekmene et al. (2009) and Philippe et al. (2003). The addition of 10 mmol/kg water $CaCl_2$ was observed to lower

the pH of milk from 6.70 to 6.33 (**Figure 5c**), which was in line with the literature data (Philippe et al., 2003). The decrease in pH also contributes to an increase in the concentrations and activities of free Ca^{2+} and Mg^{2+} ions in serum. However, the model calculation was 0.15 pH unit higher than the measured pH results at the highest level of added $CaCl_2$. Though the predicted trend follows the experimental results, we are not yet able to explain this deviation.

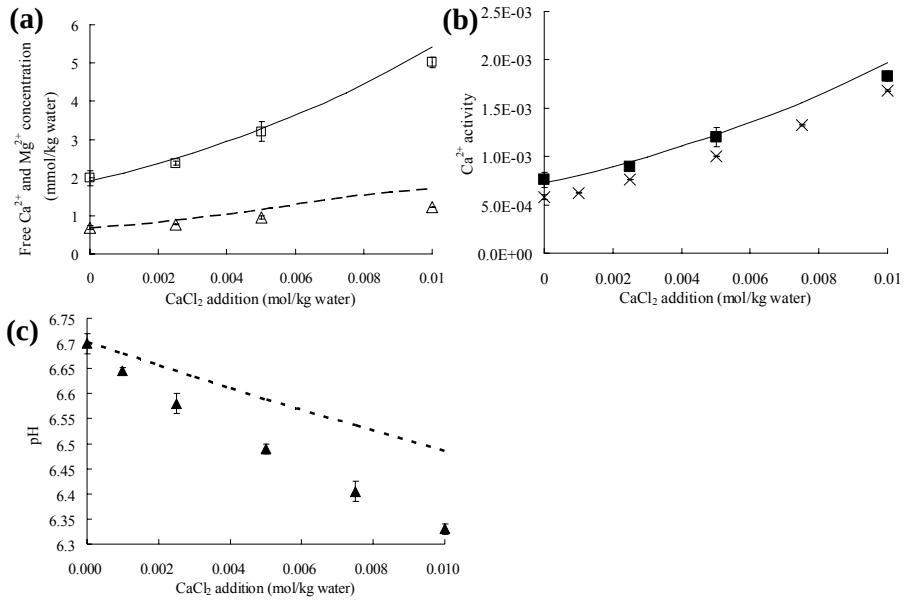

Figure 5. The influence of added $CaCl_2$ on ion equilibria of milk. (a) Effect of addition of $CaCl_2$ on free Ca^{2+} and Mg^{2+} concentration: (□) Ca^{2+} concentration measured by DMT; (—) Calculated Ca^{2+} concentration; (△) Mg^{2+} concentration measured by DMT; (– –) Calculated Mg^{2+} concentration. (b) Ca^{2+} activity as a function of added $CaCl_2$ concentration: (■) DMT method; (×) Ca-ISE; (—) Calculated Ca^{2+} activity. (c) Effect of added $CaCl_2$ on the pH of milk: (▲) measured by pH electrode; (---) Model calculation.

The ion distribution of milk after addition of $CaCl_2$ was calculated as shown in **Figure 6**. In general, micellar Ca and P_i as well as the serum Ca increase, but the serum P_i decreases as $CaCl_2$ is added to milk. After addition of 10 mmol/kg water $CaCl_2$, the changes in the concentration of micellar Ca and P_i and serum Ca and P_i are +5.2, +4.4, +4.8 and -4.4 mmol/kg water, respectively (**Figure 6**). The model suggests that part of the added calcium precipitates the serum inorganic phosphate to preferentially form $CaHPO_4 \cdot 2H_2O$ since the milk is saturated with respect to calcium phosphate. The basic form $Ca_4(PO_4)_2(OH)_2$ slightly decreases and the casein-bound Ca slightly increases. Our model also suggests that part of the added calcium remained in the free form and in the ion complex form such as $CaCitrate^-$ in the serum phase. The calculation does not predict a reduction of $citrate^{3-}$ ions in serum phase, whereas Tessier & Rose (1958) and Philippe et al. (2003) reported a reduction of 1.3 mM and 0.5 mM $citrate^{3-}$ ions, respectively, in serum phase with addition of 10 mM $CaCl_2$. It seems that the $citrate^{3-}$ ions might be saturated by the calcium ions. The discrepancy between the model and literature results is most likely due to the solubility product of $Ca_3Citrate_2$ used in the model. The model suggests the $citrate^{3-}$ ions are still not saturated by Ca^{2+} ions after addition of $CaCl_2$ of 10 mmol/kg water. Instead, the model did find an increase in $CaCitrate^-$ ion complex, which is in agreement with the calculated results by Philippe et al. (2003). **Figure 6** also shows a small decrease in micellar Mg of 1.2 mmol/kg water, suggesting partly due to the exchange between Ca and Mg, and partly due to a decrease in pH. Generally, the model calculation is in good agreement with literature data (Mekmene et al., 2009; Tessier & Rose, 1958; Udabage et al., 2000) in terms of Ca, Mg and P_i ions distribution.

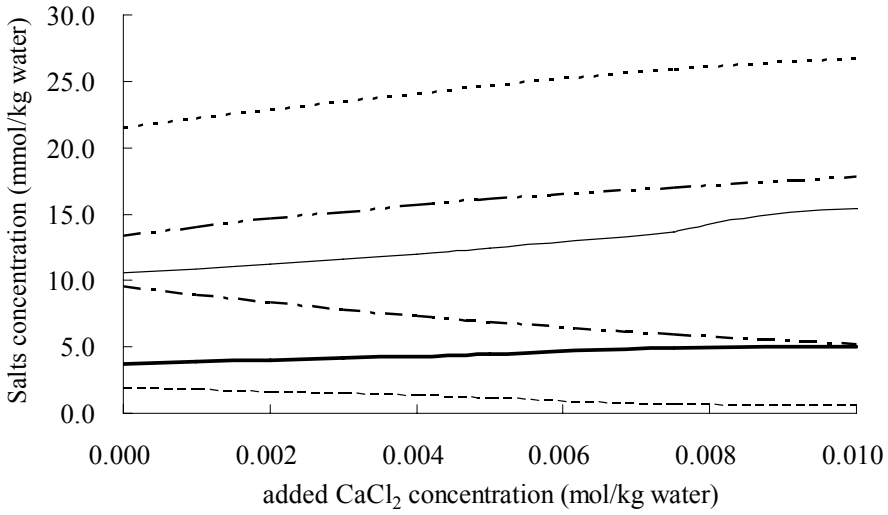

Figure 6. Model simulation of the effect of added $CaCl_2$ on major ions partition between serum and micellar phase: (—) serum Ca; (---) micellar Ca; (– - –) serum inorganic phosphate (P_i); (– --) micellar inorganic phosphate (P_i) (▬) serum Mg; (– –) micellar Mg.

6.3.5 Addition of disodium phosphate (DSP)

The addition of disodium phosphate decreased the concentrations and activities of free Ca^{2+} and Mg^{2+} ions and increased the pH of milk (**Figure 7**). The addition of 0.6 % DSP led to a decrease of free Ca^{2+} concentration from 2.05 to 0.54 mmol/kg water, and Mg^{2+} concentration from 0.68 to 0.19 mmol/kg water (**Figure 7a**). Similarly, the free Ca^{2+} activity decreased from 7.58E-04 to 1.74E-04, and the pH increased from 6.70 to 7.11 after addition of 0.6 % DSP (**Figure 7b** and **7c**). Addition of DSP also increased the ionic strength of milk from 80 to 143 mmol/kg water (**Table 6**). The calculated results were generally in line with the experimental results (**Figure 7**), although the calculated pH had a deviation (< 0.1 pH unit) from the

experimental results at the higher concentrations. Furthermore, a comparison of our results was made with literature results. For instance, Udabage et al. (2000) mentioned that the free Ca^{2+} activity was 3.3E-04 after addition of 30 mM phosphate at pH 6.65. Vujicic et al. (1968) reported that the pH of skim milk increased to 7.08 after addition of 0.5 % of disodium phosphate. Mekmene et al. (2009) calculated that the ionic strength increased from 73 to 109 mM after 20 mM DSP was added. In general, the experimental and calculated results were in line with these literature data.

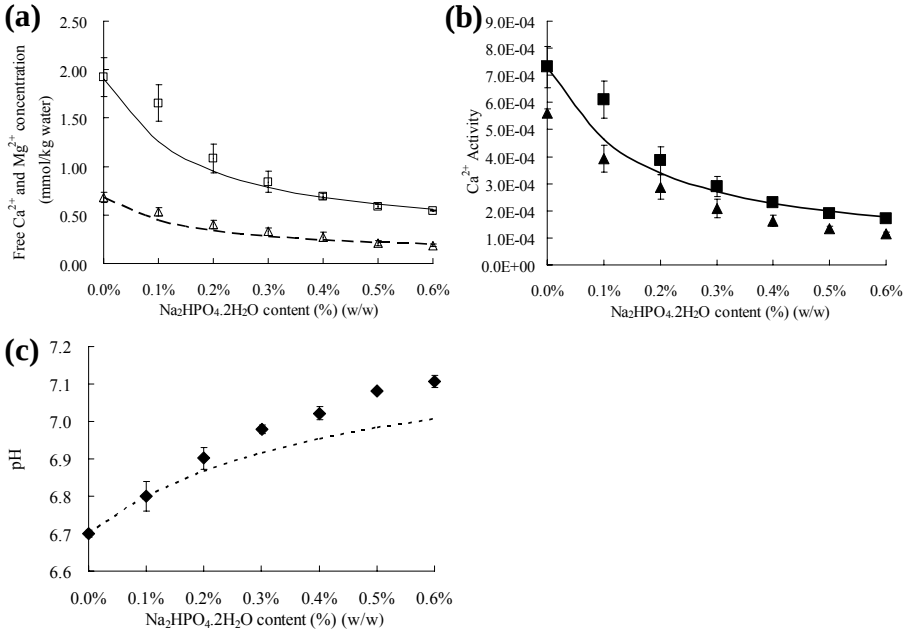

Figure 7. Effect of added $Na_2HPO_4 \cdot 2H_2O$ on ion equilibria of milk. (a) Effect of addition of $Na_2HPO_4 \cdot 2H_2O$ on free Ca^{2+} and Mg^{2+} concentration: (□) Ca^{2+} concentration measured by DMT; (—) Calculated Ca^{2+} concentration; (△) Mg^{2+} concentration measured by DMT; (– –) Calculated Mg^{2+} concentration. (b) Ca^{2+} activity as a function of added $Na_2HPO_4 \cdot 2H_2O$ concentration: (■)

DMT method; (▲) Ca-ISE; (—) Calculated Ca^{2+} activity. (c) Effect of added $Na_2HPO_4 \cdot 2H_2O$ on the pH of milk: (◆) measured by pH electrode; (---) Model calculation.

The model calculation shows not only the concentrations of free Ca^{2+} and Mg^{2+}, but also the concentrations of serum Ca and Mg decrease upon addition of DSP (**Figure 8**). It is suggested that the addition of phosphate precipitates the free Ca^{2+} and Mg^{2+} ions, thereby resulting in an increase of calcium phosphate and magnesium phosphate in colloidal calcium phosphate. However, some researchers (Udabage et al., 2000) reported that the serum Mg did not change after addition of DSP. The discrepancy can be explained by the difference in pH between the literature data (Udabage et al., 2000) and the present study. The milk samples were kept constant pH at 6.65 in the literature, while the pH of milk samples increased from 6.70 to 7.11 in our study. The more alkaline environment promotes the formation of solid magnesium phosphate. Furthermore, the model shows that the casein-bound Ca and Mg tend to decrease, suggesting the competition between inorganic phosphate and casein phosphoserine residues. **Figure 8** also shows that most of the added phosphate remains in the serum phase, which is in accordance with literature results (Gaucher et al., 2007; Mekmene et al., 2009; Udabage et al., 2000; Vujicic et al., 1968).

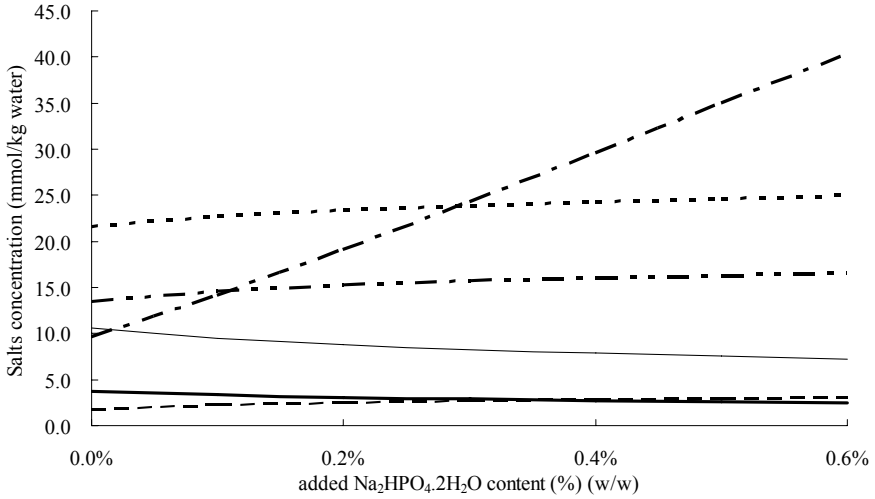

Figure 8. Model simulation of the effect of added $Na_2HPO_4 \cdot 2H_2O$ on major ions partition between serum and micellar phase: (—) serum Ca; (---) micellar Ca; (– - –) serum inorganic phosphate (P_i); (– --) micellar inorganic phosphate (P_i) (▬) serum Mg; (– –) micellar Mg.

6.4 Conclusion

The milk ion speciation (MIS) model has been shown to give satisfactory predictions of ion equilibria in milk and milk enriched with different electrolytes in comparison with our experimental results and literature data, even though the pH prediction is less accurate. One important characteristic of the MIS model is that the formulation of the colloidal calcium phosphate is defined as a mixture of acidic and basic type of calcium phosphate, which distinguishes it from the composition of CCP in the previous models. Another feature is that the MIS model calculates the ionic composition of milk at different pH values, resulting in a more realistic simulation closer to the practical situations. Moreover, a very interesting feature revealed by the

model is that there is a sharp transition range in CCP in between pH 6.59 and 6.77, which still requires further research. However, the MIS model still has some limitations. This model is for the moment only validated at room temperature. It is not yet capable to describe the ion equilibria at different temperatures due to lack of association constants and solubility products. Another limitation is that the MIS model does not include all milk components. Constituents, such as casein carboxyl groups, and micellar citrate are not included. The absence of these compounds may induce an error in predicting the buffering capacity and interactions between cations and proteins, but the results of model calculation do not appear to influence the concentrations and activities of calcium and magnesium ions at various conditions. Nevertheless, with the available model, it becomes possible to explain and to predict behaviour of ions in milk systems. By implementing this model, a significant step can be made in transferring fundamental knowledge to the R&D environments of dairy industry, but also that of the food industry in general. There, this knowledge can be applied in new product development, such as new types of cheese and new formulations of liquid dairy products, and also can be used to deliver solutions to problems in existing dairy products.

Acknowledgement

This work is funded by FrieslandCampina Innovation Europe. The authors are grateful to Arie vanden Berg and Peter Nobels from the Department of Soil Quality, Wageningen University, for performing ICP-AES measurement. We would also like to thank MSc student Radhika Bongoni and Faridah for performing part of experimental work.

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Chapter 7

Accurate determination of the Ca^{2+} activity in milk-based system by Ca-ISE: effects of ionic composition on the single Ca^{2+} activity coefficient and liquid junction potentials

This Chapter has been submitted as:
Gao, R., van Leeuwen, H. P., van Valenberg, H. J. F., & van Boekel, M. A. J. S. Accurate determination of the Ca^{2+} activity in milk-based system by Ca-ISE: effect of ionic composition on the single Ca^{2+} activity coefficient and liquid junction potentials.

Abstract

Calcium ion selective electrode (Ca-ISE) was found to underestimate the actual Ca^{2+} ion activity in simulated milk ultrafiltrate (SMUF) and milk. It is shown that the ionic compositional difference between conventional calibration solutions and milk type samples had a significant effect on the single Ca^{2+} activity coefficient, which generates the erroneous estimate of Ca^{2+} activities in SMUF and milk. This study tests new standards with ionic profiles similar to SMUF, aiming at the reduction of the errors generated by the compositional difference between conventional standards and milk samples. As a result, the new standards showed a significant improvement in the accuracy of Ca^{2+} activity and Ca^{2+} activity coefficient over the conventional standards. The systematic error is reduced from 20 % to 5 % for SMUF and from 44 % to 15 % for milk. In addition, the new standards generate liquid junction potentials that are practically insignificant.

Keywords**:** Ca-ISE, Ca^{2+} activity, Ca^{2+} activity coefficient, liquid junction potential, SMUF, skim milk

7.1 Introduction

Ionized calcium (Ca^{2+}) is well known to play a crucial role in physicochemical properties of casein micelles, such as stabilization, gelation induced by acid and rennet, heat stability, ethanol stability, surface and rheological properties (Fox & McSweeney, 1998; Walstra & Jenness, 1984). Therefore, it is of great importance in practice to accurately measure the activity of free Ca^{2+} ions in milk.

The calcium ion selective electrode (Ca-ISE) has been widely used to determine free Ca^{2+} ion activity in milk, having the advantage of speed, ease of analysis and repeatability (Fox & McSweeney, 1998; Geerts, Bekhof & Scherjon, 1983; Holt, Dalgleish & Jenness, 1981; Lin, Lewis & Grandison, 2006; Muldoon & Liska, 1969; Silanikove, Shapiro & Shamay, 2003; Tsioulpas, Lewis & Grandison, 2007). However, Holt et al. (1981) indicated that the Ca-ISE technique suffers from the disadvantage that calibration solutions do not contain all of the components of milk or milk diffusate and hence interfering substances may give rise to erroneous estimates. So far, the conventional standards, which consist of different concentrations of $CaCl_2$ and KCl, and have similar ionic strength to milk, have been used for calibration of Ca-ISE for the measurement of Ca^{2+} activity in milk systems. Looking back on the previous studies, Silanikove et al. (2003) mentioned that the presence of casein micelles could remarkably affect determination of Ca^{2+} ion activity in milk, resulting in lower Ca^{2+} ion activity by the Ca-ISE. Holt et al. (1981) indicated that the phosphate ester fraction possibly interfered with ionic calcium activity measurement. Gao et al. (2009; 2010a; 2010c) reported that the experimental Ca^{2+} ion activities and activity coefficients of simulated milk ultrafiltrate (SMUF) and reconstituted skim milk by Ca-ISE

were significantly lower than both the model calculated results and the experimental data determined by Donnan membrane technique (DMT). These problems have already been observed by Butler (1968) who demonstrated that the differences in ionic composition between calibration solutions and samples generate differences in the Ca^{2+} ion activity coefficient, even if the solutions have the same ionic strength. To overcome the problems, the calibration solutions should be as similar as possible to milk or milk serum rather than only match their ionic strength. Since still significant quantities of sodium, magnesium, phosphates, citrate, metal-anion complexes and milk proteins (casein micelles) are present in milk, they may have a significant effect on the Ca^{2+} activity coefficient. Moreover, the difference in ionic composition between the conventional standards and milk samples may also affect the liquid junction potential of the electrode, which will contribute to an erroneous estimate of the Ca^{2+} activity coefficient and the measurement of Ca^{2+} activity. More recently, Lin et al. (2006) used calcium standard solutions containing imidazole to calibrate the Ca-ISE for the measurement of Ca^{2+} activity in milk samples. However, the standard solutions still have a large difference in ionic composition from milk. That is, the error still remains in the measurement. Not much research has been carried out to develop calibration solutions similar to milk or milk serum for the measurement of Ca^{2+} activity in milk. The difficulty is to obtain the Ca^{2+} activities of the calibration solutions when these solutions have similar composition to milk or milk serum. Recently, an ion speciation model (DIS) was developed by Gao et al. (2010a; 2010b) and gave satisfactory predictions of the ion composition in freshly prepared simulated milk ultrafiltrate (SMUF). With the aid of this DIS model, the Ca^{2+} activities in calibration solutions can be calculated so that development of calibration solutions similar to milk serum may be possible.

The objective of this study is to formulate new calibration solutions for the measurement of Ca^{2+} activity in milk-like systems using Ca-ISE, with the purpose to reduce the effect of ionic composition on the Ca^{2+} activity coefficient and liquid junction potential. New calibration solutions were defined according to the composition of SMUF, thereby similar to milk serum. The Ca^{2+} ion activities measured by the new standards will be set against conventional data and evaluated in terms of the resulting Ca^{2+} ion activity coefficients and liquid junction potentials.

7.2 Materials and Methods

All experiments were carried out at 20 ± 1.0 ºC and all glassware was washed in phosphate-free detergent, soaked in 1.4 M nitric acid and rinsed in deionized water (Millipore, Amsterdam, The Netherlands). All chemicals used were purchased from Fluka, with 99.5 % purity. Simulated milk ultrafiltrate (SMUF) was prepared according to Jenness & Koops (1962). Skim milk was reconstituted with low heat skim milk powder (Nilac, NIZO, The Netherlands), as described by Gao et al.(2009).

7.2.1 Determination of free Ca^{2+} ion activity by Ca-ISE

7.2.1.1 Preparation of standards for calibration of Ca-ISE

Conventional standards and new standards were prepared for the calibration of the calcium ion selective electrode. **Table 1** shows that the new standards are formulated to have an ionic composition similar to SMUF. Each new calibration solution was composed of $CaCl_2$, KCl plus a stock solution containing K_3Citrate, Na_3Citrate, KH_2PO_4 and $MgCl_2$, as shown in **Table 1** and **2**. The major ions in SMUF are Ca, Mg, Na, K, phosphate, citrate and

chloride, the concentrations of which are 9, 3.2, 18.3, 39.2, 11.6, 9.6 and 33.5 mmol/kg water, respectively. The compositions of the new calibration solutions are in general agreement with SMUF. The procedure of preparation of new calibration solutions was briefly described as follows. The stock solution was first prepared by weighing and dissolving the four chemicals specified in **Table 2**. Subsequently, $CaCl_2$ and KCl were separately weighed and added into stock solution to constitute one calibration solution. In addition, both the conventional standards and the new standards have the same effective ionic strength with of 90 mmol/kg water.

Table 1. **C**oncentrations (mmol/kg water) of $CaCl_2$ and KCl used in conventional and new standards for calibration of Ca-ISE for milk systems.

	Calibration Standards Conventional		Calibration Standards New	
Calibration solutions	$CaCl_2$	KCl	$CaCl_2$	KCl
1	0.3	93.9	6.6	46.3
2	1.0	92.4	8.9	43.2
3	3.0	86.1	10.6	39.6
4	5.0	80.6	12.2	36.0
5	10.0	66.5	13.6	32.6

Table 2. Composition of the stock solution for new calibration solutions

Component	Concentration (mmol/kg water)
K_3Citrate	3.0
Na_3Citrate	7.0
KH_2PO_4	10.0
$MgCl_2$	3.0

7.2.1.2 Determination of free Ca^{2+} activity in SMUF and skim milk

Free Ca^{2+} activity was measured using an Orion 720A+ meter (Thermo, Beverly, USA) equipped with a calcium ion selective electrode (Orion 97-20 ion plus, Thermo, Beverly, USA). Calibration was carried out with freshly prepared standard solutions in the range between 10^{-4} and 10^{-2} mol/kg water $CaCl_2$. The effective ionic strength for each calibration solution was kept constant, as mentioned at 90 mmol/kg water. The Ca^{2+} ion activity of each calibration solution was theoretically calculated using the dynamic ion speciation model (DIS) (section 7.2.2). The calibration curve was obtained by plotting the measured potential (mV) as a function of theoretically calculated calcium activity. The time necessary to reach a stable reading was approximately 5 min, referring to electric potential change less than 0.1 mV per minute.

7.2.1.3 Ca^{2+} activity coefficient ($\gamma_{Ca^{2+}}$)

The Ca^{2+} activity coefficients of SMUF and milk were calculated using the following equation

$$\gamma_{Ca^{2+}} = \frac{a_{Ca^{2+}}}{m_{Ca^{2+}}} \quad (1)$$

where $a_{Ca^{2+}}$ and $m_{Ca^{2+}}$ are the measured activity and concentration of Ca^{2+} ions of SMUF or milk, respectively.

Moreover, the Ca^{2+} activity coefficient can be calculated based on the Davies equation as follows:

$$\log(\gamma_{Ca^{2+},Davies}) = -0.5 \cdot (z_{Ca^{2+}})^2 \cdot \left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.2I\right) \quad (2)$$

where $\gamma_{Ca^{2+},Davies}$ refers to the Ca^{2+} activity coefficient in a solution containing

only $CaCl_2$, z_{Ca}^{2+} is the charge of calcium ions and I is the effective ionic strength.

According to Butler (1968), the ionic composition will influence the single ion activity coefficient in spite of constant ionic strength for different solutions. Butler determined the mean activity coefficients of $CaCl_2$ in the solution containing various concentrations of NaCl and $CaCl_2$. Based on the assumption of Guggenheim, the single Ca^{2+} activity coefficient was expressed as follows:

$$\gamma_{Ca^{2+},Butler} = (\gamma_{\pm(CaCl_2)})^2 \quad (3)$$

where $\gamma_{Ca^{2+},Butler}$ represents the single Ca^{2+} activity coefficient in NaCl-$CaCl_2$ solution and $\gamma_{\pm(CaCl2)}$ is the mean activity coefficient of $CaCl_2$ in the NaCl-$CaCl_2$ solution. When the NaCl-$CaCl_2$ solution had an ionic strength of 0.07 mol/kg water, the $\gamma_{\pm(CaCl2)}$ was shown to be 0.62 (Butler, 1968). The resulting $\gamma_{Ca^{2+},Butler}$ was then compared with $\gamma_{Ca^{2+},Davies}$ and the experimental activity coefficient ($\gamma_{Ca^{2+}}$), as shown in the section 7.3.2.

7.2.2 Liquid junction potential (E_j)

The Ca-ISE (Orion 97-20 ion plus, Thermo, Beverly, USA) employs a salt bridge and reference electrode of fixed potential. The potential of the Ca-ISE is given by

$$E_{Ca} = \text{constant} + \frac{RT}{2F}\ln(m_{Ca^{2+}}) + \frac{RT}{2F}\ln(\gamma_{Ca^{2+}}) + E_j \quad (4)$$

where $m_{Ca^{2+}}$ is the concentration of free Ca^{2+} ion, $\gamma_{Ca^{2+}}$ is the activity coefficient of free Ca^{2+} ion, and E_j is the diffusion potential resulting from the liquid junction between the salt bridge solution and the test solution. To estimate the liquid junction potential (E_j), the Henderson equation (Eq. (5)) was employed (Fry & Langley, 2001)

$$E_j = \frac{\sum_i \frac{|z_i| u_i}{z_i}\left[c_i(2) - c_i(1)\right]}{\sum_i |z_i| u_i \left[c_i(2) - c_i(1)\right]} \cdot \frac{RT}{F} \ln \sum_i \frac{|z_i| u_i c_i(1)}{|z_i| u_i c_i(2)} \quad (5)$$

where u_i, z_i and c_i are the mobility, valence and concentration of ion i, $c_i(1)$ and $c_i(2)$ are the molar concentration of ion i in the salt bridge solution and sample solution, respectively, and $|z_i|$ is the modulus of z_i, which is always positive.

When calculating the E_j generated from the contact between salt bridge solution and SMUF, the concentrations of the major ions and ion pairs/complexes were obtained as computed by the DIS model (Gao et al., 2010a) and the mobilities of the major ions and ion complexes were taken from literature (Bazinet, Castaigne & Pouliot, 2005; Fry & Langley, 2001). The same procedure was applied for the liquid junction potentials with the conventional standards and new standards. In addition, the E_j obtained between bridge solution and SMUF can also be regarded as E_j between bridge solution and skim milk, since SMUF represents the ionic composition of milk serum.

7.2.3 DIS model

The DIS model has been shown to satisfactorily predict the ion composition in freshly prepared SMUF solution (Gao et al., 2010a; Gao et al., 2010b). Briefly, all the major components of SMUF (Ca, Mg, Na, K, chloride, citrate, phosphate, sulphate and carbonate) are included in the DIS model. The components are supposed to react with each other, resulting in the formation of free ions, ion pairs or complexes, and solid precipitates. Moreover, the model includes a pool of association constants and solubility products, and mass balance equations for each component. The resulting equations

describing ion speciation are nonlinear algebraic equations that were solved numerically to obtain activities, concentrations and activity coefficient for all the components and their complexes. In this paper, the DIS model was used to calculate the free Ca^{2+} ion activities and concentrations in both conventional and new standards.

7.3 Results and Discussions

7.3.1 The calibration standards for the Ca-ISE

The conventional standards for calibration of Ca-ISE have a free Ca^{2+} concentration ranging from 0.1 to 10.0 mmol/kg water, as shown in **Table 3**. The model calculation shows that the free Ca^{2+} concentration of each calibration solution is lower than the concentration of $CaCl_2$ used for the corresponding calibration solution (**Table 1**), which is due to the formation of $CaCl^+$ ion pairs. Similarly, the free Ca^{2+} concentration varies from 1.0 to 5.0 mmol/kg water in the new standards, which is much lower than the total calcium concentration used for the new standards (**Table 1**). This is because a large fraction of calcium ions forms ion complexes with citrate, phosphate and chloride ions. The pH values of the new calibration solutions were calculated and compared with the measured pH values. **Table 3** shows that the DIS model provides a satisfactory prediction in pH of the new standards, which implies the calculated Ca^{2+} activities and concentrations are quite reliable.

Table 3. The calculated concentrations (m_{Ca}^{2+}) (mmol/kg water) and activities (a_{Ca}^{2+}) of free Ca^{2+} ion, and pH of the calibration solutions for Ca-ISE [a].

	Calibration Standards Conventional		Calibration Standards New			
Calibration Solutions	m_{Ca}^{2+}	a_{Ca}^{2+}	m_{Ca}^{2+}	a_{Ca}^{2+}	pH_{Calc}	pH_{Meas} (SD)[b]
1	0.25	9.36E-05	1.0	3.72E-04	5.92	5.92 (0.02)
2	0.84	3.12E-04	2.0	7.40E-04	5.81	5.82 (0.01)
3	2.54	9.41E-04	3.0	1.11E-03	5.75	5.75 (0.02)
4	4.26	1.58E-03	4.0	1.48E-03	5.70	5.71 (0.02)
5	8.54	3.16E-03	5.0	1.85E-03	5.66	5.68(0.02)

[a] The free Ca^{2+} concentration and activity, and pH_{Calc} of each calibration solution are calculated by the DIS model. pH_{Meas} is the experimental values, measured by a pH electrode.

[b] n=3.

7.3.2 The effect of calibration standards on Ca^{2+} activity measurement

The calcium ion selective electrode was calibrated separately with the conventional standards and new standards. Calibration curves were obtained by plotting electric potential (E_{Ca}) versus the calculated calcium activities (-log a_{Ca}^{2+}). **Figure 1** displays that the electrode is generally well calibrated in both standards with the slope 29.6 mV for the conventional standards and 29.3 mV for the new standards. It appeared that the conventional calibration curve was nearly in parallel with the new calibration curve, but with an intercept gap of 7.4 mV. Regarding the measurement of Ca^{2+} activity in milk, the E_{Ca} of milk had a mean value of 36.9 mV using the conventional standards, and a mean value of 31.8 mV using new standards. Moreover, the E_{Ca} of milk serum obtained using the new standards, was 32.5 mV on average.

The E_{Ca} values were subsequently converted into Ca^{2+} activity and expressed as $a_{Ca^{2+},C}$ for milk using conventional standards, $a_{Ca^{2+},N}$ for milk using new standards and $a_{Ca^{2+},N}^{*}$ for milk serum using new standards as shown in **Figure 1**. All the values of $a_{Ca^{2+},C}$, $a_{Ca^{2+},N}$ and $a_{Ca^{2+},N}^{*}$ of milk are given in **Table 4**, which displayed an order $a_{Ca^{2+},N}^{*} > a_{Ca^{2+},N} > a_{Ca^{2+},C}$. The same applies for Ca^{2+} activity in SMUF solution, as shown in **Table 4**.

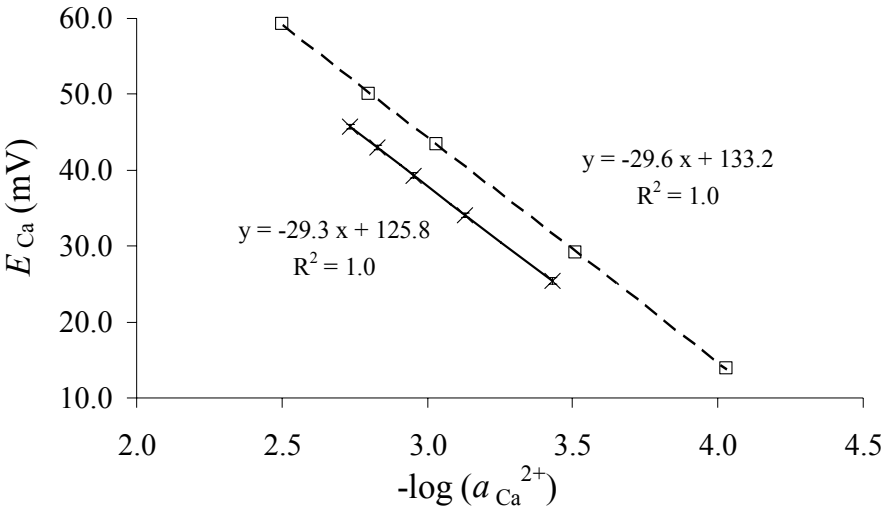

Figure 1 Calibration curves for Ca-ISE: (□) represents conventional calibration standards, and (×) represents the new calibration standards. The symbols a and b, respectively, represent the Ca^{2+} activity of milk serum ($a_{Ca^{2+},N}^{*}$) and milk ($a_{Ca^{2+},N}$) determined by the new calibration standards; the symbol c represents the Ca^{2+} activity of milk ($a_{Ca^{2+},C}$) measured by the conventional standards.

Table 4 also shows the various Ca^{2+} activity coefficients of SMUF and skim milk: $\gamma_{Ca^{2+},C}$ and $\gamma_{Ca^{2+},N}$ represent the $\gamma_{Ca^{2+}}$ for SMUF or skim milk obtained by the conventional standards and the new standards, respectively, and

$\gamma_{Ca^{2+},N}^{*}$ represents the $\gamma_{Ca^{2+}}$ of milk serum obtained by the new standards. The activity coefficients $\gamma_{Ca^{2+},N}$ and $\gamma_{Ca^{2+},N}^{*}$ were significantly higher than the $\gamma_{Ca^{2+},C}$ for both SMUF and skim milk. However, significant differences still remain between $\gamma_{Ca^{2+},N}$ and $\gamma_{Ca^{2+},\text{Davies}}$ or between $\gamma_{Ca^{2+},N}^{*}$ and $\gamma_{Ca^{2+},\text{Davies}}$ (**Table 4**). A question is therefore raised on the accuracy of the $\gamma_{Ca^{2+},\text{Davies}}$ for SMUF and skim milk. Butler (1968) reported that the mean activity coefficient of $CaCl_2$ in a solution mixed with NaCl and $CaCl_2$ (experimental data) was lower than it was in a solution of the same ionic strength but containing only $CaCl_2$ (calculated data according to the modified Debye-Hückel equation by Guggenheim) when the ionic strength was below 0.3 mol/kg water. As a result, the single Ca^{2+} ion activity coefficient ($\gamma_{Ca^{2+},\text{Butler}}$) in the NaCl-$CaCl_2$ solution was found to be 8 % lower than the value of $\gamma_{Ca^{2+},\text{Davies}}$ at the ionic strength similar to SMUF or skim milk (Butler, 1968), as shown in **Table 4**. This indicates that there is an error in the estimation of the Ca^{2+} activity coefficient in the NaCl-$CaCl_2$ solution by the Davies equation even though no difference in ionic strength exists between the NaCl-$CaCl_2$ solution and the solution containing only $CaCl_2$. Thus, it makes sense that Ca^{2+} activity coefficients of SMUF and skim milk differ from the $\gamma_{Ca^{2+},\text{Davies}}$. The presence of many components may cause a larger difference in Ca^{2+} activity coefficient between SMUF or skim milk and the $\gamma_{Ca^{2+},\text{Davies}}$. Moreover, the $\gamma_{Ca^{2+},N}$ and $\gamma_{Ca^{2+},N}^{*}$ were found in satisfactory agreement with $\gamma_{Ca^{2+},\text{Butler}}$, particularly for SMUF (**Table 4**). This reveals that the individual ion activity coefficient is sensitive to ionic composition, rather than overall ionic strength which is a too global parameter.

Table 4. The Ca^{2+} activity ($a_{Ca^{2+}}$) (SD) and Ca^{2+} activity coefficient ($\gamma_{Ca^{2+}}$) (SD) of SMUF and skim milk determined by conventional standards, new calibration standards and by theoretical calculation.

	$a_{Ca^{2+}}$ [a]			$m_{Ca^{2+}}$ [b]	$\gamma_{Ca^{2+}}$ [c]				
	$a_{Ca^{2+},C}$	$a_{Ca^{2+},N}$	$a_{Ca^{2+},N}^{*}$	(mol/kg H_2O)	$\gamma_{Ca^{2+},C}$	$\gamma_{Ca^{2+},N}$	$\gamma_{Ca^{2+},N}^{*}$	$\gamma_{Ca^{2+},Davies}$	$\gamma_{Ca^{2+},Butler}$
SMUF	5.4E-04 (1.5E-05)	5.8E-04 (1.2E-05)	-	1.58 (0.03)	0.34 (0.01)	0.37 (0.01)	-	0.41	0.38
Skim Milk	5.6E-04 (2.3E-05)	6.2E-04 (1.0E-05)	-	2.02 (0.06)	0.28 (0.01)	0.30 (0.01)	-	0.40	0.37
Milk Serum	-	-	6.5E-04 (1.8E-05)	2.02 (0.06)	-	-	0.32 (0.01)	-	-

[a] $a_{Ca^{2+},C}$ and $a_{Ca^{2+},N}$ represents the measured Ca^{2+} activity of SMUF or skim milk using conventional standards (n = 25) and new standards (n = 10), respectively. $a_{Ca^{2+},N}^{*}$ represents the measured Ca^{2+} activity of milk serum using new standards (n = 10).

[b] $m_{Ca^{2+}}$ represents the concentration of free Ca^{2+} ions. The values of free Ca^{2+} concentrations were taken from the literature, in which the concentrations of free Ca^{2+} ions of SMUF and skim milk were determined by Donnan Membrane technique. (Gao et al., 2009)

[c] $\gamma_{Ca^{2+},C}$ and $\gamma_{Ca^{2+},N}$ represent the Ca^{2+} activity coefficient of SMUF or skim milk using conventional standards (n = 25) and new standards (n=10), respectively. $\gamma_{Ca^{2+},N}^{*}$ represents the Ca^{2+} activity coefficient of milk serum using new standards (n = 10). These activity coefficients were calculated according to the equation (1). $\gamma_{Ca^{2+},Davies}$ represents the Ca^{2+} activity coefficient in a solution only containing $CaCl_2$, which is calculated by the Davies equation. $\gamma_{Ca^{2+},Butler}$ is the Ca^{2+} activity coefficient in a solution containing $CaCl_2$ and NaCl, having an ionic strength similar to SMUF or skim milk.(Butler, 1968)

The conventional standards contain only Ca, K and Cl ions, which have an ionic strength similar to that of SMUF or skim milk. However, the conventional standards lead to substantially erroneous estimations of Ca^{2+} activity. It can be seen that the errors are for SMUF 20 % and for milk 44 % by comparing $a_{Ca^{2+},C}$ with $a_{Ca^{2+},Davies}$ (**Tables 4** and **5**). The $a_{Ca^{2+},Davies}$ represents the Ca^{2+} activity calculated using the Ca^{2+} concentration ($m_{Ca^{2+}}$) (**Table 4**) multiplying by the activity coefficient ($\gamma_{Ca^{2+},Davies}$) based on the Davies equation. The error is due to the difference in ionic composition between the standards and the samples, which causes the calcium electrode suffering from a so-called matrix effect (Holt et al., 1981; Neville, Zhang & Allen, 1995). This means that other components in SMUF or skim milk, such as calcium complexing anions (phosphate and citrate), macromolecules (casein micelles and whey proteins) and lactose, are likely to interfere with the measurement of Ca^{2+} activity. The new standards resemble SMUF or milk serum. This largely reduces the errors generated from the ionic compositional differences between the calibration standards and sample solution (**Table 4**). Thus, **Table 5** shows the difference between $a_{Ca^{2+},N}$ ($a_{Ca^{2+},N}^{*}$) and $a_{Ca^{2+},Davies}$ is 12 % and 24 % for SMUF and milk serum, respectively. However, $a_{Ca^{2+},Davies}$ may not be the suitable reference to be compared since the $\gamma_{Ca^{2+},Davies}$ does not represent the calcium activity coefficient in SMUF or milk serum. The $\gamma_{Ca^{2+},Butler}$ is better suited to simulate the calcium activity coefficient in SMUF or milk serum since it describes the non-ideality of Ca^{2+} ions in a mixture solution. As a result, the Ca^{2+} activity $a_{Ca^{2+},Butler}$ is obtained using the Ca^{2+} concentration ($m_{Ca^{2+}}$) (**Table 4**) multiplying by the activity coefficient ($\gamma_{Ca^{2+},Butler}$) as shown in **Table 5**. Thus, the Ca^{2+} activity $a_{Ca^{2+},N}$ ($a_{Ca^{2+},N}^{*}$) by the new standards is in general agreement with the $a_{Ca^{2+},Butler}$ for SMUF (< 5 % difference), while a relative large difference still exists for skim milk (< 15 % difference). It can be concluded that the new standards significantly

improved the accuracy of Ca^{2+} activity measurement in milk-like systems.

Table 5 Comparison of Ca^{2+} activity of SMUF and skim milk determined by the conventional, the new calibration standards to the theoretical calculation [a, b].

	$a_{Ca^{2+},Davies}$	$\frac{a_{Ca^{2+},Davies}}{a_{Ca^{2+},C}}$	$\frac{a_{Ca^{2+},Davies}}{a_{Ca^{2+},N}}$	$a_{Ca^{2+},Butler}$	$\frac{a_{Ca^{2+},Butler}}{a_{Ca^{2+},N}}$
SMUF	6.5E-04	1.20	1.12	6.0E-04	1.03
Skim milk	8.1E-04	1.44	1.24	7.4E-04	1.14

[a] The $a_{Ca^{2+},Davies}$ and $a_{Ca^{2+},Butler}$ mean the calcium activity calculated by using the calcium concentration $m_{Ca^{2+}}$ (**Table 4**) multiplying calcium activity coefficient $\gamma_{Ca^{2+},Davies}$ and $\gamma_{Ca^{2+},Butler}$, respectively (**Table 4**).

[b] The $a_{Ca^{2+},N}$ for skim milk in this table is the value of calcium activity of milk serum indicated as $a_{Ca^{2+},N}^{*}$ in **Table 4**.

7.3.3 Liquid junction potential (E_j)

Liquid junction potentials arise when two solutions of different composition come into contact. A concentrated equitransference electrolyte solution containing KCl and KNO_3 is used as salt bridge solution in this Ca-ISE, which may lead to no significant changes in E_j. **Table 6** shows the calculated liquid junction potentials of the Ca-ISE in contact with calibration solutions or sample solutions. The liquid junction potentials are small and generally in the same order, all of which are below 1 mV. Moreover, the liquid junction potential appears not to change significantly from the calibration solution to the sample solution. This indicates that the liquid junction potential of the Ca-ISE is rather stable, and hence will not generate significant errors in the measurement of Ca^{2+} activity in milk systems.

Table 6 The calculated liquid junction potential E_j (mV) of Ca-ISE in calibration standards and SMUF [a].

	E_j (mV)
Conventional standards	0.60
New standards	0.70
SMUF	0.53

[a] The E_j is calculated by Henderson equation (Eq.(5))

7.4 Conclusions

The results for the proposed new calibration solutions have demonstrated the great importance of calibrating the Ca-ISE in solutions with compositions similar to those of the samples. The new standards remarkably improve the accuracy of determination of Ca^{2+} activity in milk-like systems by Ca-ISE. Their features are summarized as follows:

i) The compositional difference between calibration solutions and sample solution has large influence on estimation of Ca^{2+} activity coefficient and hence on Ca^{2+} activity. The new standards have ionic profiles nearly the same as SMUF and similar to milk serum. The difference in Ca^{2+} activity or activity coefficient between the measured data and the actual value is 5 % for SMUF, and 15 % for milk. Further improvement on determining Ca^{2+} activity in milk, if necessary, should be possible by including components such as lactose and phosphate ester in even more dedicated calibration standards.

ii) The effect of ionic composition on the liquid junction potential appears not significant within random error of ± 0.2 mV. The liquid junction potential of Ca-ISE remains stable due to well controlled composition of salt bridge solution for the reference electrode.

iii) The dynamic ion speciation (DIS) model appears to be helpful in developing new calibration solutions for calibration of Ca-ISE to determine Ca^{2+} ion activity in milk-like systems. Ion speciation models thus show their power, provided they have been validated extensively, as we have done in previous work. While we have used milk-like systems in this work, it is anticipated that this approach is also helpful for other systems with a complex ion composition.

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Chapter 8

General Discussion

8.1 Introduction

The behaviours of ions in milk and milk-derived systems at different conditions have been extensively studied in this thesis. The ion equilibria of milk-like systems, particularly the concentrations of free Ca^{2+} and Mg^{2+} ions, have been successfully measured by the Donnan Membrane Technique (DMT). DMT has shown its advantages of good accuracy, no distortion of original equilibria and simultaneous determination of free metal ions. However, DMT also shows relatively long time span to obtain Donnan equilibrium compared to the other technique of the calcium ion selective electrode (Ca-ISE). Moreover, the Ca-ISE measurement is performing well when using the right calibration standards (**Chapter 7**). The Ca-ISE method was found to underestimate the Ca^{2+} activity in milk-like systems when using the simple prescribed standards, due to the so-called matrix effect. The ionic compositional difference between the conventional standards and the measured milk-like systems appeared to induce unexpected deviations in the Ca^{2+} activity coefficient and hence on Ca^{2+} activity. With development of new standards that have ionic composition close to milk serum, the deviations generated by the ionic composition difference are largely reduced. Besides the quantification of ion equilibria, ion speciation models have been developed to predict the ion behaviours in milk-like systems, namely equilibrium ion speciation (EIS), dynamic ion speciation (DIS) and milk ion speciation (MIS). The models have been demonstrated to give satisfactory predictions of ion composition in milk-like systems enriched with different electrolytes. In addition, sugars were observed to substantially influence ion activities in milk-like systems. Rather than using the well-known Debye-Hückel theory, the more recent developed Mean Spherical Approximation (MSA) theory has been successfully applied to explaining the

effects of sugars on ion behaviours. The MSA theory suggests that the excluded volume effects mainly account for a large increase in ion activity coefficient, thereby causing an increase in ion activity. MSA theory has shown its ability to describe ion properties in practical concentrated systems.
In this thesis, the main objective has been to develop ion speciation models to describe ion equilibria in milk-like systems. This chapter will focus on evaluation of the ion speciation models on the one hand, and a case study of applying the developed ion speciation models to cheese on the other hand to challenge and test the predictive capability of the models.

8.2 Evaluation of the ion speciation models

8.2.1 Equilibrium ion speciation (EIS) model and dynamic ion speciation (DIS) model

As shown in **Chapters 3 and 4**, both EIS and DIS model give satisfactory prediction of ion compositions in simulated milk ultrafiltrate (SMUF) at different conditions. The EIS model is a thermodynamic ion speciation model, which reveals the ion composition of SMUF at equilibrium. The DIS model on the other hand describes the ion composition in freshly prepared SMUF. The advantages of the DIS model are: (i) simulation of ion composition of milk serum; (ii) taking into account specific ion effects (Na^+ not being equivalent to K^+); (iii) prediction of the effect of various polyphosphates on ion equilibria in SMUF. However, the DIS model was developed using an apparent solubility product for $CaHPO_4 \cdot 2H_2O$ as a function of time to tackle the calcium phosphate precipitation kinetics. It is a purely empirical approach. There are many physico-chemical reactions happening during calcium phosphate precipitation, such as crystal nucleation, crystallization and transformation between precipitates. For instance, Van Kemenade and De

Bruyn (1987) reported that the calcium phosphate precipitation sequence below pH 6.7 starts from octacalcium phosphate (OCP) followed by DCPD ($CaHPO_4 \cdot 2H_2O$). The DIS model does not describe such a transformation of the two precipitates. Hence, a logical follow-up would be to study precipitation kinetics in more detail to be able to turn the empirical DIS model into a mechanistic model. If that is achieved, the DIS model can not only calculate the ion composition of fresh SMUF, but also can simulate the changes in ion equilibria of SMUF on its way to equilibrium. Such a model is needed and relevant because of calcium phosphate precipitation occurring in several processes, e.g., the fouling problem due to such precipitation is of great practical importance in dairy industry.

8.2.2 Milk Ion Speciation (MIS) Model

The MIS model deals with the ion equilibria between the salts present in the aqueous phase and micellar phase of milk, as described in **Chapter 6**. The MIS model has shown to give satisfactory prediction of ion composition in milk upon addition of different electrolytes. Very recently, Mekmene et al. (2009, 2010) presented two models to simulate the ion equilibria of milk at fixed pH and upon acidification, respectively. The models gave reasonable predictions of ion equilibria compared to experimental results, though differences in the order of 10 to 20 % still remain between model and experimental results. The main differences between Mekmene's model and the MIS model are, in the first place, the MIS model is a generic model because it describes the ion equilibria of milk at various conditions. Secondly, a difference remains in the assumption of the nature of CCP. In the MIS model, CCP is considered to be a more basic type of calcium phosphate since the molar ratio of micellar Ca/micellar P_i is 1.84 for milk at neutral pH. In Mekmene's models, two molar ratios of Ca/P_i are used: 1.4 (Mekmene et al.,

2009) and 1.83 (Mekmene et al., 2010), implying that the nature of CCP varies in the two models. Moreover, magnesium is considered to exist in the micellar phase as $Mg_3citrate_2$ by Mekmene et al. (2010), which is an incorrect assumption since Mg is not present in the form of $Mg_3citrate_2$ as reported by Alexander and Ford (1957). Consequently, the MIS model appears better to describe the ion equilibria of milk under various conditions since it is a generalized model and the assumption of the nature of CCP sounds more reasonable. However, the MIS model has some disadvantages, such as neglecting of citrate in the micellar phase and association between cations and amino acids with carboxyl groups of casein proteins ($CN\text{-}RCOO^-$). To solve the first problem, full understanding of the nature of the colloidal calcium phosphate is required, while for the second problem, the used software program AESolve reached its limits in terms of calculating capacity when including association between cations and $CN\text{-}RCOO^-$ groups. Moreover, the effects of addition of polyphosphates on ion compositions of milk systems cannot be completely described by the model either. Being able to do that is of great practical relevance to understand physico-chemical properties of products, such as processed cheese, sweetened condensed milk, and UHT milk. That is because the mechanism of interactions between polyphosphates and salts in milk is not completely known yet, as discussed in this chapter and possible solutions are proposed.

8.2.2.1 The nature of colloidal calcium phosphate (CCP)

Until now, no solid experimental results have shown the exact composition of colloidal calcium phosphate. In the MIS model, the colloidal calcium phosphate is defined as a mixture of acidic and basic calcium phosphate phases, but tends to have more basic characteristics. This is based on several facts:

(i) The molar ratio of micellar Ca to the micellar inorganic phosphate (Ca/P_i) is often used to determine the nature of CCP. The experimental molar ratio Ca/P_i is 1.58 in the pH range 5 – 6.7 if we assume, for the moment, that the depicted relation is linear (**Figure 1**). Particularly, the Ca/P_i ratio has a mean value of 1.84 ± 0.10 at pH 6.7 (**Chapter 6**). This is in general agreement with literature results reported by McGann et al. (1983) and Dalgleish and Law (1989).

(ii) Lucey and Horne (2009) suggested that the form of CCP is a basic form based on various titration studies.

(iii) At pH near 6.70 different calcium phosphate phases may actually coexist because at this pH phase transition occurs for acidic and more basic calcium phosphates (Van Kemenade & De Bruyn, 1987).

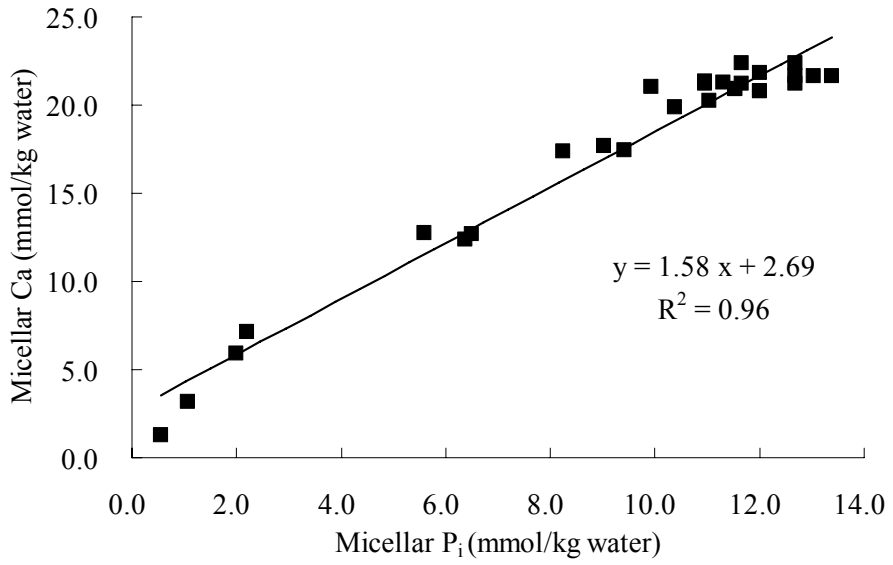


Figure 1 Concentrations of micellar Ca and micellar P_i in milk in the pH range 5.0 to 6.7 at 20 °C. The line is obtained via linear regression and the

slope represents the Ca/P_i molar ratio in micellar calcium phosphate.

Therefore, CCP is considered to contain $Ca_4(PO_4)_2(OH)_2$, $CaHPO_4$ and $Mg_3(PO_4)_2$ in the MIS model. As **Chapter 6** showed, the modeled results with such composition of CCP were generally in line with experimental results. However, differences still remain between calculated results and experimental data in solubilization of CCP, particularly for $Mg_3(PO_4)_2$ and micellar P_i, during acidification and addition of NaCl or KCl. In the first place, the MIS model suggests that $Mg_3(PO_4)_2$ is totally solubilized at pH 6.59, which is not in accordance with literature data (Le Graët & Brule, 1993). Second, the model shows that addition of NaCl or KCl induces complete solubilization of $Mg_3(PO_4)_2$, whereas literature showed only a slight increase in serum Mg (Le Graët & Brule, 1993). This suggests that the form of $Mg_3(PO_4)_2$ used in the model is not completely correct, which may affect the accuracy of model predictions in Mg, Ca and P_i partitioning. Apart from the difficulties with $Mg_3(PO_4)_2$, the model shows that the concentrations of micellar P_i and serum P_i have irregular behaviours between pH 6.77 and 6.59 during acidification (**Figure 2 in Chapter 6**). The model suggests that a transition between $CaHPO_4$ and $Ca_4(PO_4)_2(OH)_2$ occurs in this pH range. This finding shows the power of modelling: such a transition over a narrow pH range would probably have gone unnoticed otherwise. However, whether it is a model artifact or a real phenomenon requires further validation. Consequently, such questions about the actual composition of CCP need to be addressed to be sure that the model gives accurate predictions of in ion partitioning in milk.

From the activities of Ca^{2+}, Mg^{2+}, $citrate^{3-}$ and phosphate ions calculated by the MIS model, we could obtain the ion activity products of insoluble compounds that are probably present in the micellar phase. According to

Chaplin (1984), if the ions in the aqueous phase are in an equilibrium with those of the solid phase, the ion activity product should be constant, independent of pH, and a graph of -log (ion activity product) should be a horizontal line. First of all, it is evident that the ion activity product of each solid compound is not constant throughout the pH range from 5.0 to 8.0 (**Figure 2**). This indicates that CCP does not have an invariant composition in this pH range. Second, the acidic calcium phosphate $CaHPO_4$ exhibits constant ion activity products in the pH range 5.0 to 6.8. On the contrary, the basic calcium phosphate $Ca_5(PO_4)_3(OH)_2$, $Ca_4(PO_4)_2(OH)_2$ and $Ca_3(PO_4)_2$ show constant ion activity products in the pH range from 6.6 to 8.0. Interestingly, there is an overlapping pH region 6.6–6.8 for the ion activity products of the acidic and the basic calcium phosphates. This suggests that it is indeed likely to have the acidic and basic forms coexisting in CCP between pH 6.6 and 6.8, in which a phase transition between calcium phosphates occurs. In contrast, Holt (1982) reported that the Ca/P_i ratio was not affected by the pH within the range 4.8–8.4, suggesting that the micellar calcium phosphate is uniform in composition in this pH range. However, the nature of CCP cannot only be judged based on the Ca/P_i ratio. A mixture of calcium phosphates may also have a constant Ca/P_i ratio at different pH. Moreover, the plot shown in **Figure 1** does not seem to be completely linear, particularly at high or low concentration of micellar Ca and P_i, implying that the composition of CCP may, in fact, change with pH. Therefore, it is reasonable to assume basic and acidic calcium phosphates existing in CCP. We propose that the basic form has a molecular structure similar to hydroxylapatite ($Ca_5(PO_4)_3(OH)_2$), such as $Ca_4(PO_4)_2(OH)_2$ used in the MIS model. This is in agreement with literature data by Lyster (1976) who reported that micellar calcium phosphate resembled hydroxylapatite to some extent. The acidic form should be $CaHPO_4$ since another acidic form, octacalcium phosphate

($Ca_4H(PO_4)_3$), should be excluded in CCP due to its changing ion activity product throughout the pH range (**Figure 2**). Third, magnesium phosphates behave similarly as calcium phosphates in the micellar phase. The acidic form $MgHPO_4$ is preferably present in the acidic pH range 5.0–6.8, while the basic form $Mg_3(PO_4)_2$ prefers to stay in the more basic pH range 6.6–8.0. As mentioned earlier, the model does not give satisfactory prediction of the change in magnesium in milk by using $Mg_3(PO_4)_2$. This suggests that it would be more reasonable to use the acidic form of magnesium phosphate ($MgHPO_4$) in the model. Moreover, the model also suggests that an ion activity product is constant in the range 5.0–6.8 for a molecular formula $Ca_{0.83}Mg_{0.17}HPO_4$ (**Figure 2**). It makes sense to have such a formula since Mg is partly associated with CCP as reported by Holt (1985). As pH increases, the acidic magnesium phosphate will be converted to the basic form $Mg_3(PO_4)_2$. Last but not least, micellar citrate was found to be associated exclusively with CCP (Holt, 1985). $Ca_3Citrate_2$ will be the most probable form present in CCP since no magnesium citrate was found in the micellar phase (Alexander & Ford, 1957). **Figure 2** shows that the ion activity product of $Ca_3Citrate_2$ remains nearly constant below pH 6.7. In summary, the most realistic composition of CCP used in the MIS model is that $Ca_4(PO_4)_2(OH)_2$, $CaHPO_4$, $MgHPO_4$ and $Ca_3Citrate_2$. An alternative composition is a mixture of $Ca_4(PO_4)_2(OH)_2$, $Ca_{0.83}Mg_{0.17}HPO_4$ and $Ca_3Citrate_2$. These suggestions can be further tested and compared to experimental results.

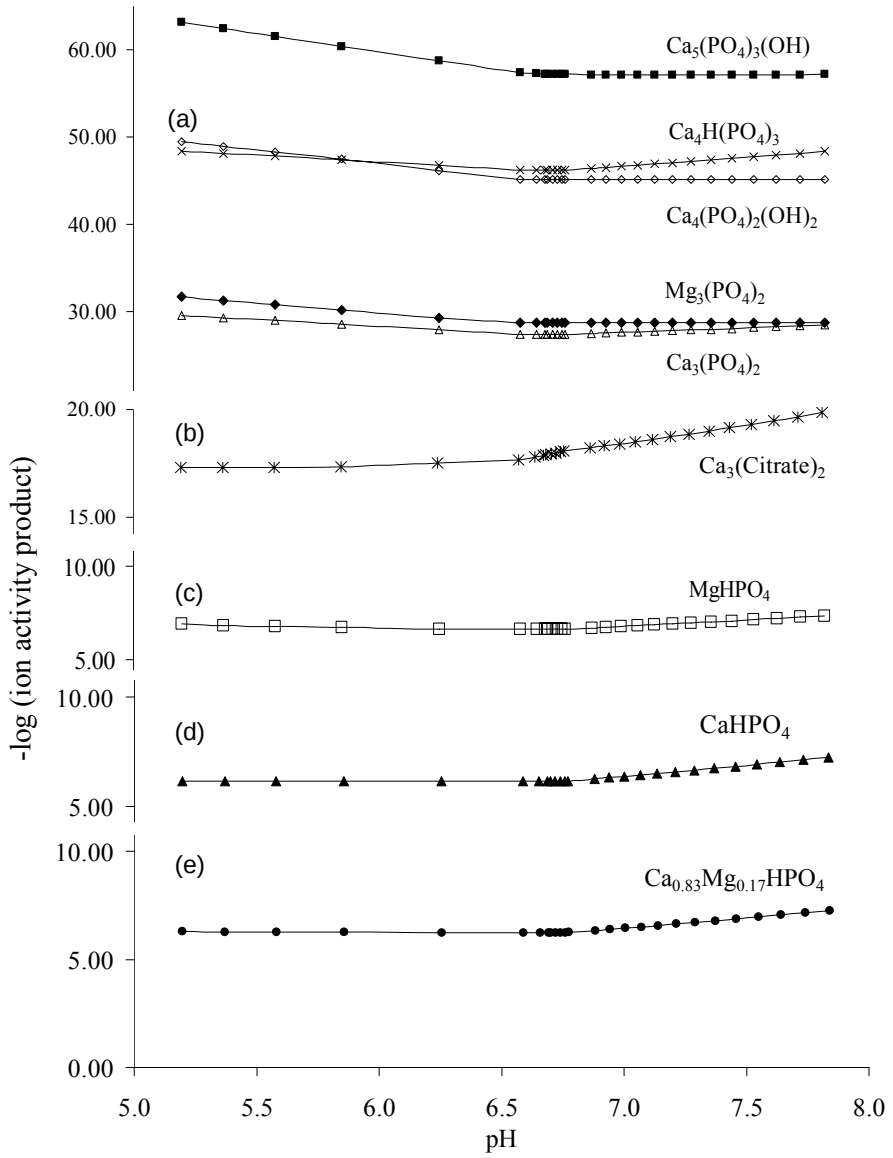


Figure 2. The dependence of –log (ion activity product) for (a) hydroxylapatite ($Ca_5(PO_4)_3OH$), octacalcium phosphate ($Ca_4H(PO_4)_3$), $Ca_4(PO_4)_2(OH)_2$, amorphous trimagnesium phosphate ($Mg_3(PO_4)_2$), amorphous tricalcium phosphate ($Ca_3(PO_4)_2$); (b) tricalcium citrate

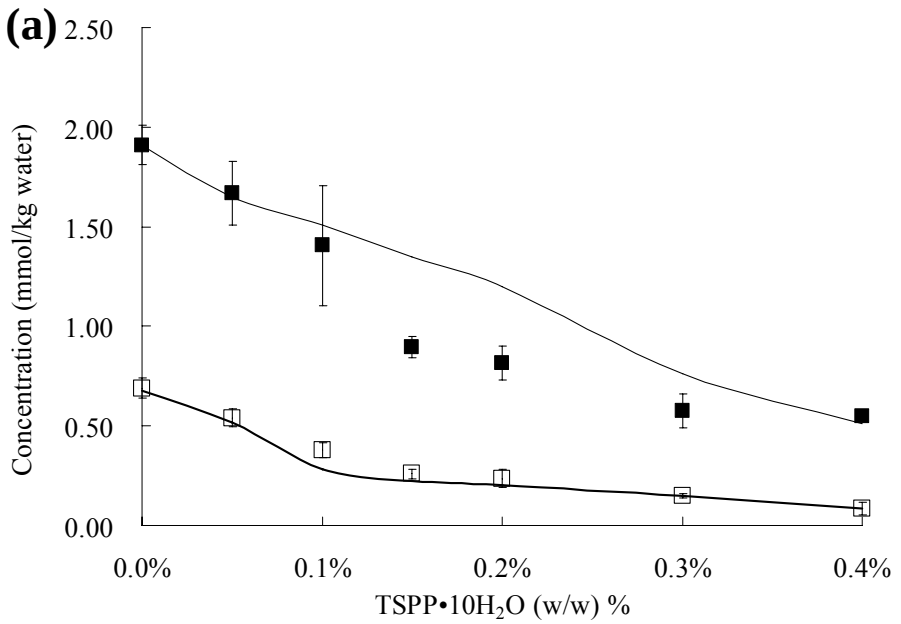
($Ca_3Citrate_2$); (c) $MgHPO_4$; (d) $CaHPO_4$ and (e) $Ca_{0.83}Mg_{0.17}HPO_4$ in milk on pH. Values are calculated from the ion equilibria in milk by the MIS model.

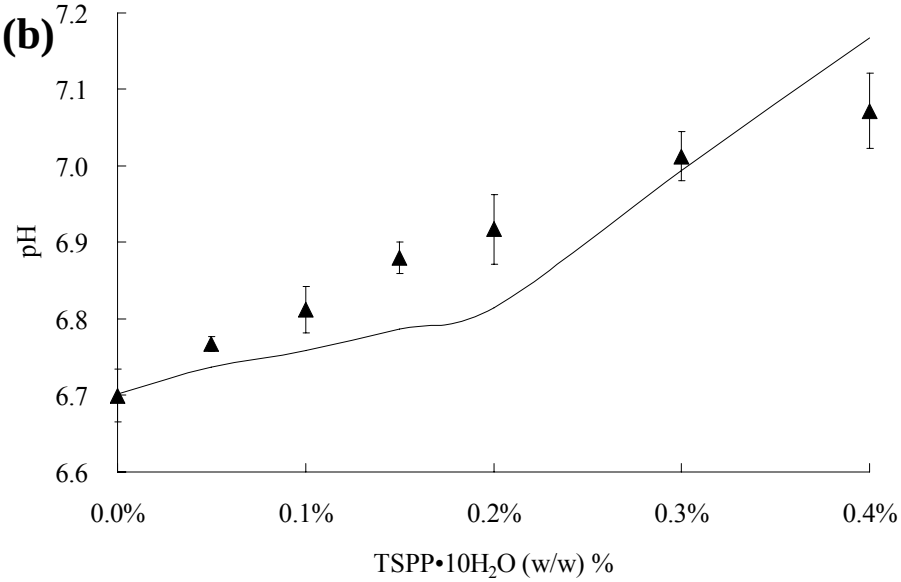
8.2.2.2 Effect of addition of tetrasodium pyrophosphate (TSPP)

In milk, TSPP ($Na_4P_2O_7 \cdot 10H_2O$) can complex free Ca^{2+} and Mg^{2+} ions, and can further form solid precipitates with Ca^{2+} and Mg^{2+} ions if sufficient pyrophosphate is present. As the concentration of TSPP increased from 0 to 0.4 %, the concentrations of free Ca^{2+} and Mg^{2+} ions decreased from 1.91 to 0.55 mmol/kg water for Ca^{2+} ions and from 0.69 to 0.08 mmol/kg water for Mg^{2+} ions (**Figure 3a**). The pH of milk increased from 6.70 to 7.08 as shown in **Figure 3b**. Moreover, **Figure 3c** shows that addition of TSPP changes the ion partitioning in milk. Interestingly, the concentrations of serum Ca and inorganic phosphate (P_i) initially increased and then decreased over the range of added TSPP. This is different from the observed phenomena in SMUF with added TSPP, where the total Ca and P_i decreased throughout the whole range of TSPP because of the formation of solid precipitate $Ca_2P_2O_7$ (**Chapter 3**). This indicates that TSPP induces changes in the equilibria between the colloidal calcium phosphate and dissolved Ca and P_i ions. Mizuno and Lucey (2007) studied the formation of TSPP induced-gelation from milk protein concentrate (MPC) solutions. The authors proposed a possible gelation mechanism, in which the addition of TSPP induces dispersion of casein micelles, followed by the formation of calcium pyrophosphate ($Ca_2P_2O_7$). As a result, the calcium pyrophosphate may crosslink caseins to facilitate protein gelation. However, excessive TSPP concentration weakens the gelation due to an increase in electrostatic repulsion via calcium pyrophosphate (Mizuno & Lucey, 2007). Our experimental results of the changes of the concentrations of serum Ca and P_i with added TSPP in milk appeared to be in line with this mechanism. Initially, addition of TSPP disperses the casein

micelles because TSPP can complex the soluble Ca^{2+} ions in milk serum, leading to solubilization of colloidal calcium phosphate. As a result, the concentrations of serum Ca and P_i increase. As the TSPP concentration increases, the Ca^{2+} ions may form solid calcium pyrophosphate. The latter can crosslink casein proteins, resulting in a decrease in serum Ca and P_i.

The effect of addition of TSPP to milk was also simulated by the MIS model (**Figure 3**). All the relevant association constants (**Chapter 2**) were employed in the MIS model. The solubility products of calcium pyrophosphate and magnesium pyrophosphate were included as well. The model calculation is in line with experimental results in terms of free Ca^{2+} and Mg^{2+} concentrations and the serum Na concentration (**Figure 3a and c**). However, the model does not give satisfactory prediction in pH, serum Ca and P_i (**Figure 3b and 3c**), but it does follow the trend. The measured pH increased nearly linearly, while the model shows a non-linear relationship between pH and added TSPP. The calculated serum Ca shows a similar trend to the measured results, but it significantly departures from the experimental data. Also, the calculated serum P_i increases in the range of added TSPP, which is different from experimental results. An attempt to change the solubility product of $Ca_2P_2O_7$ did not lead to improvement. We cannot yet give a satisfactory explanation for the discrepancy between the model calculation and experimental results. We suggest that it is related to the nature of CCP, and that further testing with the model needs to be done using the CCP composition suggested in section 8.2.2.1.


(a)
2.50
2.00
1.50
1.00
0.50
0.00
Concentration (mmol/kg water)
0.0%
0.1%
0.2%
0.3%
0.4%
TSPP•10H2O (w/w) %


(b)
7.2
7.1
7.0
6.9
6.8
6.7
6.6
pH
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0.1%
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0.3%
0.4%
TSPP•10H2O (w/w) %

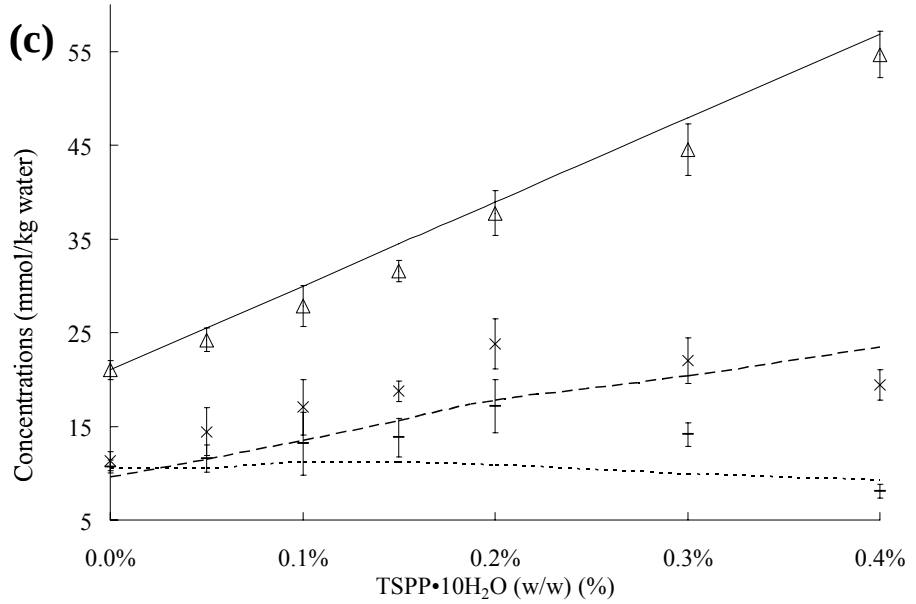

Figure 3. Effect of addition of TSPP on ion composition in milk (n=3). **(a)** (■) free Ca^{2+}, (□) measured free Mg^{2+} (—) MIS model; **(b)** (▲) pH, (—) MIS model; **(c)** (△) Serum Na, (—) Calculated Na, (×) Serum inorganic phosphate (P_i), (– –) Calculated P_i, (-) Serum Ca, (---) Calculated Ca

8.3 Application of the models in simulation of ion equilibria in cheese

8.3.1 Calculation of ion composition in one-month old cheddar cheese juice

The pH and mineral content have a major influence on the structure and texture of cheese (Everett & Auty, 2008; Johnson & Lucey, 2006; Lucey & Fox, 1993; Lucey et al., 2005). Rather than the total Ca content, the proportion of colloidal calcium phosphate (CCP) plays an important role in controlling the functionality of cheese (Johnson & Lucey, 2006; Lucey & Fox,

1993). For cheeses with similar total Ca concentration, the concentration of undissolved calcium phosphate of the two cheeses may be significantly different due to different pH or different aging stages, thereby resulting in different functional properties, such as melting, stretching and firmness. Therefore, the dynamic calcium ion equilibria between undissolved Ca and dissolved Ca are crucial in relation to the functionality of cheese. By using an ion speciation model, we are able to simulate the ion composition in cheese in order to correlate the changes of calcium equilibria with cheese functionality during cheese ripening. The dynamic ion speciation (DIS) model was used for two reasons. One reason is that cheese juice is similar to simulated milk ultrafiltrate (SMUF), which is supersaturated with calcium phosphate. Second, the DIS model can simulate the changes of ion composition during aging of cheese. The experimental mineral composition of one-month Cheddar cheese juice was taken from Morris et al. (1988) for this simulation study.

Table 1 shows the calculated ion composition of one-month old Cheddar cheese juice by the DIS model. The divalent cations, especially the dissolved Ca mainly exists in the form of $CaLactate^{+}$, $CaCl^{+}$ and free Ca^{2+} ions. There are also several undissolved Ca compounds present. The DIS model suggests that three solid compounds are formed in cheese juice, namely $CaHPO_4$, $Ca_3Citrate_2$ and $CaLactate_2$ (**Table 1**). This is based on the observation that the calculated ion activity products of all the three compounds give horizontal lines in the pH range 5–6 (**Figure 4**). These solid compounds were also experimentally identified in cheese juice by Morris et al. (1988). Apparently, our modeled results are in line with literature. Besides, the concentrations of $CaHPO_4$, $Ca_3Citrate_2$ and $CaLactate_2$ are calculated to be 247, 12.9 and 6.3 mmol/kg of water, respectively for the one-month old

cheese juice (**Table 1**). The modeled results were compared to literature data by Morris et al. (1988) (**Table 1**), who used the model of Holt et al. (1981) to calculate the concentrations of dissolved ion species and pH of the same sample. It turns out that our modeled results are in general agreement with those calculated by Morris et al. (1988), which shows the predictive capacity of the DIS model.

Table 1 Calculated concentrations (mmol/kg water) of ions and complexes in one-month old cheddar cheese juice by the DIS model [a] versus literature values from Morris et al. (1988) [b] indicated between brackets.

		Cation complex			
Anion	Free ion	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
$Citrate^{3-}$	- (0.3)	3.6 (11.1)	0.5 (1.1)	- [c] (-)	0.2 (0.2)
$HCitrate^{2-}$	0.1 (0.3)	0.3 (0.6)	- (-)	- (-)	0.6 (0.5)
HPO_4^{2-}	0.6 (0.3)	0.9 (0.5)	0.2 (0.1)	0.1 (0.1)	1.7 (0.9)
$H_2PO_4^{-}$	18.8 (19.2)	2.4 (2.2)	0.4 (0.4)	0.4 (0.5)	5.3 (6.5)
$Glc\text{-}1\text{-}PH^{-}$	16.8 (11.1)	- (1.1)	- (0.2)	- (0.3)	- (3.8)
$Glc\text{-}1\text{-}P^{2-}$	1.6 (1.7)	1.2 (1.4)	0.2 (0.2)	0.1 (0.1)	2.1 (2.0)
HLactate	7.6 (10.2)	- (-)	- (-)	- (-)	- (-)
$Lactate^{-}$	331 (337.7)	86.5 (80.5)	16.8 (15.2)	4.4 (5.3)	59 (69.2)
Cl^{-}	638 (610)	36.5 (58.4)	11.5 (5.4)	13.6 (14.6)	180.2
SO_4^{2-}	1.1 (1.1)	0.5 (0.6)	0.1 (0.1)	0.1 (0.1)	1.0 (0.9)
HAcetate	2.0 (1.5)	- (-)	- (-)	- (-)	- (-)
$Acetate^{-}$	11.2 (6.3)	1.9 (1.0)	- (0.2)	- (0.1)	- (1.5)
Free ion	-	44.8 (49.7)	7.1 (5.8)	54.2 (51.8)	719 (681)

[a] The total concentration (mmol/kg water) of ions used in the DIS model calculation was taken from Morris et al. (1988) in one-month old cheddar cheese juice, in which [Ca] = 471, [Mg] = 36.7, [Na] = 972, [K] = 72.8, [P_i] = 278, [Cl] = 880, [Citrate] = 31.1, [SO_4] = 2.8, [Lactate] = 518, [Acetate] =

15.1, [Glc-1-P] = 22.0. The DIS model suggests that various solid precipitates are formed, which are DCPD = 247 mmol/kg water, $Ca_3Citrate_2$ = 12.9 mmol/kg water, and $Ca(Lactate)_2$ = 6.3 mmol/kg water. The calculated ionic strength is 1.088 mol/kg water, and the calculated pH is 5.24.

[b] pH of cheese juice was measured to be 5.23 by Morris et al..

[c] Concentration shown as: - < 0.01 mmol/kg water

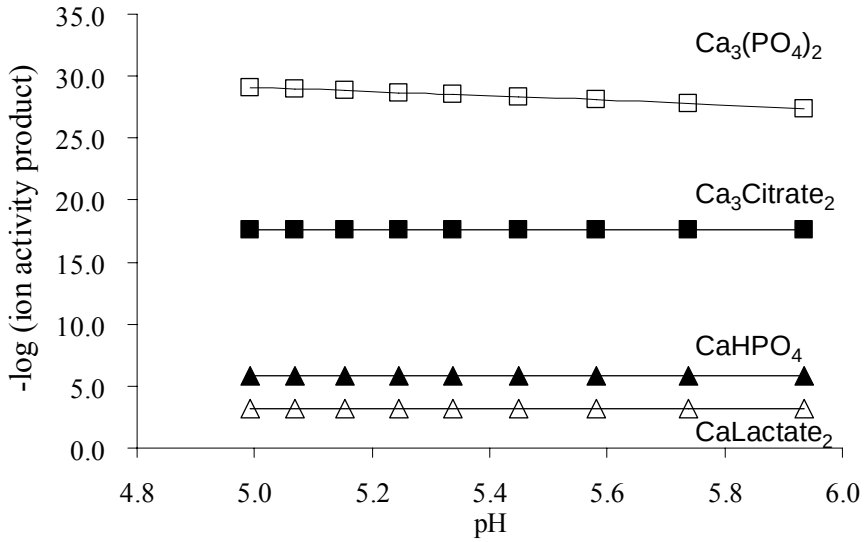

Figure 4. The dependence of –log (ion activity product) for amorphous tricalcium phosphate ($Ca_3(PO_4)_2$), tricalcium citrate ($Ca_3Citrate_2$), $CaHPO_4$ and $CaLactate_2$ in one-month old cheddar cheese juice on pH. Values are calculated from the ion equilibria in the cheese juice by the DIS model.

8.3.2 Simulation of the effect of pH on ion equilibria in cheese juice

Ion equilibria will change during the ripening of cheese due to the pH-induced solubilization of colloidal calcium phosphate and ongoing biochemical processes. The influence of pH (pH range 5–6) on the mineral

composition in one-month old cheese juice was simulated by the DIS model as shown in **Figure 5** (keeping all other factors unchanged). The model suggests that the undissolved $CaHPO_4$ decreases as pH drops, while the solid $Ca_3Citrate_2$ is hardly influenced by pH (**Figure 5a and b**). **Figure 5c** shows that the concentration of free Ca^{2+} ions increases as pH decreases, while that of free Mg^{2+} ions remains independent of pH as suggested by the model. Moreover, **Figure 6** shows that the proportion of undissolved Ca in the one-month old cheese juice decreases from 65 % to 58 % of the total cheese Ca as pH is decreased from 6 to 5. Hassan and Lucey (2001) measured the proportion of undissolved Ca in cheddar cheese juice, which decreased from 64 % to 56 % of total cheese Ca during the first 4 weeks. This indicates that the model calculation of the proportion of undissolved Ca is in agreement with literature data for one-month old cheddar cheese. A reduction in the ratio of undissolved Ca to dissolved Ca leads to an increase in cheese meltability (Everett & Auty, 2008). Applying Horne's dual-binding model (Horne, 1998), a reduction in pH decreases the amount of CCP crosslinking with casein particles. This leads to a decrease in the casein-casein interaction and an increase in electrostatic repulsion, which can weaken the cheese structure and increase cheese meltability (Lucey et al., 2003). Therefore, we can apply this model to explain difference in meltability and firmness between Cheddar cheese and Gouda cheese. Gouda cheese (pH 5.8) has a higher pH than Cheddar cheese (pH 5.2). The proportion of undissolved Ca should be higher in Gouda cheese than in Cheddar cheese, leading to stronger casein-casein interaction in the former. The model was also applied to calculate the amount of undissociated lactic acid which plays an important role in inhibiting the growth of *Listeria Monocytogenes* in Gouda cheese. The model suggests that the amount of lactic acid decreases from 11.9 to 1.3 mmol/kg of water as pH increases from 5 to 6. The minimum content of undissociated lactic acid

needed to inhibit *Listeria Monocytogenes* growth is 3.6-5.7 mmol/kg of water (Coroller et al., 2005). At the pH of Gouda cheese (pH 5.5), lactic acid concentration was calculated to be 4.1 mmol/kg water. This implies that the growth of *Listeria Monocytogenes* in Gouda cheese may be inhibited, but we cannot exclude the risk since the calculated concentration is much close to the lower concentration range proposed by Coroller et al. (2005). Moreover, the presence of other acids such as acetic acid and citric acid may have the same inhibiting effect. We recommend full studies of the mineral composition of Gouda cheese juice and development of dedicated microbial growth models for the growth of *Listeria Monocytogenes* and our developed DIS model can be helpful in this respect.

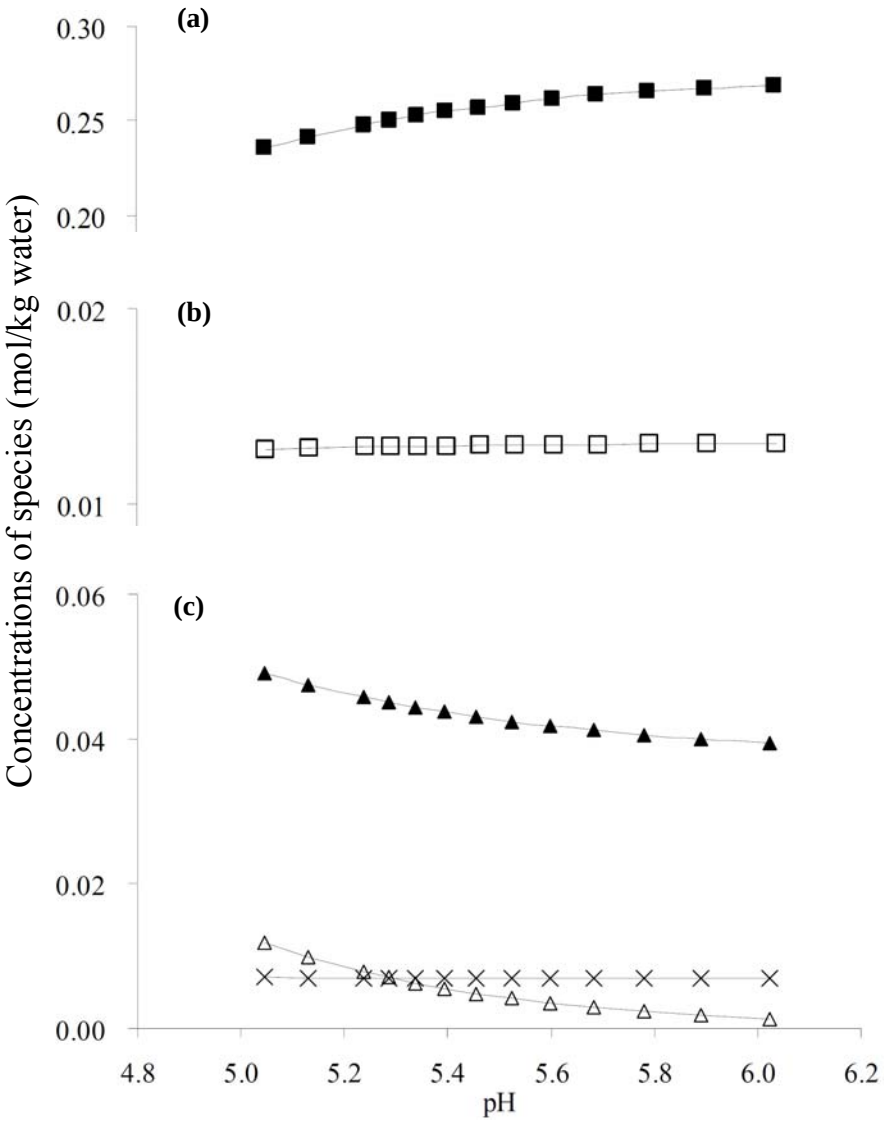

Figure 5. **M**odel calculation of ion composition as a function of pH in one-month old Cheddar cheese juice. (a) (■) solid $CaHPO_4$; (b) (□) solid $Ca_3Citrate_2$; (c) (▲) free Ca^{2+}, (×) Mg^{2+}, (△) Lactic acid.

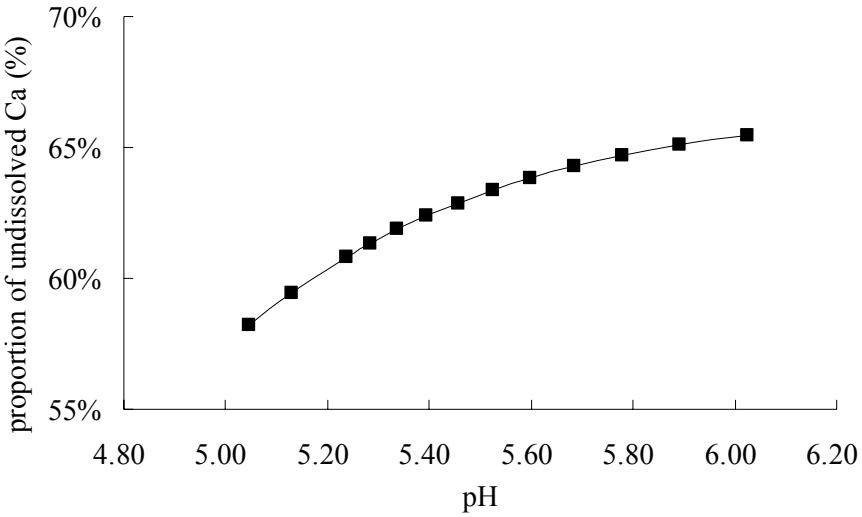

Figure 6. Proportion of undissolved Ca (%) in one-month old Cheddar cheese juice as a function of pH as calculated by the DIS model.

8.3.3 Simulation of $CaLactate_2$ crystal formation in cheese juice

Calcium lactate ($CaLactate_2$) crystal formation on the surface of aged Cheddar cheese is a widespread problem for the cheese industry (Kubantseva et al., 2004). Ca^{2+} ions and $lactate^-$ may form ion complexes $CaLactate^+$. If there is sufficient amount of Ca^{2+} and lactate ions, exceeding their solubility product, crystallization may occur (Kubantseva et al., 2004). It was attempted to simulate the $CaLactate_2$ crystallization in one-month old cheese juice by using the DIS model (keeping other factors unchanged). The negative logarithm of solubility product (K_{sp}) of $CaLactate_2$ for its crystalline form is 4, which is taken from literature (Walstra & Jenness, 1984). Apparent solubility products ($K_{sp,\ app}$) of $CaLactate_2$ were employed in the model to mimic the kinetic process of calcium lactate from amorphous form to crystalline form as shown in **Figure 7**. This is the same approach as described in **Chapter 3** for

simulation of the kinetics of calcium phosphate precipitation in SMUF. The model suggests that the nucleation of $CaLactate_2$ occurs at $-\log(K_{sp,\,app})$ near 3.2. During the aging of cheese, amorphous calcium lactate will be converted to the crystalline form. That is, the solubility of calcium lactate continues to decrease. By changing the solubility products from 3.2 to 4.0, the model shows that the amount of calcium lactate increases from 6.3 to 125.2 mmol/kg of water. The increase in the concentration of $CaLactate_2$ induces a decrease in colloidal calcium phosphate ($CaHPO_4$) and free Ca^{2+} ions (**Figure 7**). Moreover, the pH increases from 5.24 to 5.45, while the ionic strength decreases from 1.088 to 0.956 mol/kg of water. Kubantseva et al. (2004) unravelled that the main factors influencing the solubility of $CaLactate_2$ are the concentration of lactate ions and temperature. Therefore, the lactose content in the cheese milk should be controlled to prevent calcium lactate formation. Meanwhile, we could also use the model to calculate the maximum amount of lactose in the cheese milk that can be used to prevent such problems.

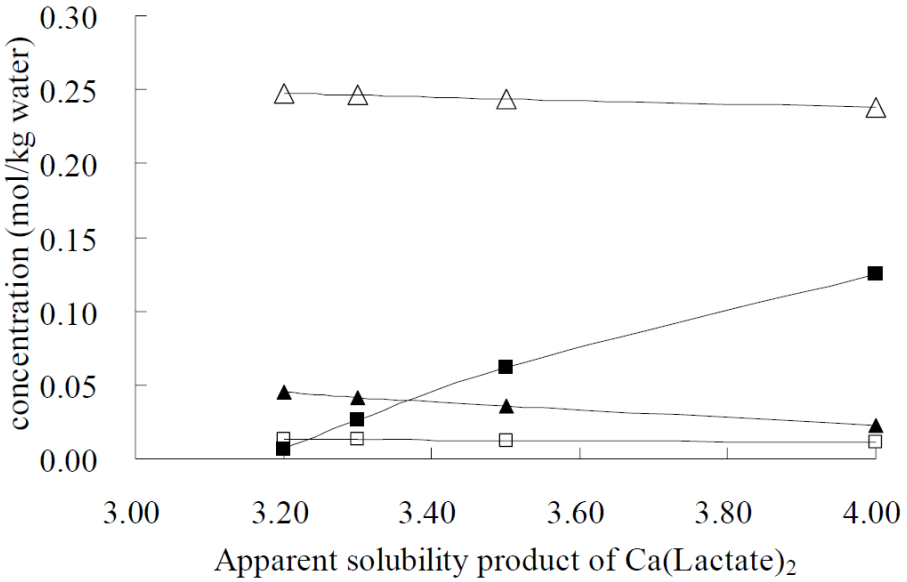

Figure 7. Simulation of the development of crystallization of $CaLactate_2$ and its effect on other species during aging of cheddar cheese by the DIS model. (△) solid $CaHPO_4$, (■) $CaLactate_2$, (▲) free Ca^{2+}, and (□) solid $Ca_3Citrate_2$. The negative logarithm of solubility product (K_{sp}) of $CaLactate_2$ crystal form is 4.0, taken from Walstra and Jenness (1984).

8.3.4 The effect of ionic strength

The Cheddar cheese juice is a concentrated system containing many electrolytes. The ionic strength of cheese juice was calculated to be 1.088 mol/kg of water (**Table 1**). Such high ionic strength may induce an error in the prediction of the value of activity coefficients of ion species for the current model based on two factors. One is the relative permittivity of the cheese juice and the other one is the commonly used Davies equation. First of all, in the current model calculation, the relative permittivity of water has been used as a constant parameter for the activity coefficient calculation. It is

still valid to use it for systems having lower ionic strength. However, systems having higher ionic strength (e.g. cheese juice) have relative permittivities significantly lower than that of pure water. Second, the Davies equation is not suitable for the concentrated systems since it does not take into account excluded volume effects. There will be an error of prediction in ion activity coefficient. In **Chapter 5**, the MSA theory was successfully applied to describe the ion properties in concentrated systems (Gao et al., 2010). The excluded volume effect accounts for the non-ideal behaviours of ion species. Therefore, a MSA model was developed to calculate the Ca activity coefficient in more concentrations systems. Due to the complexity of cheese juice, we simplified it to be a simple solution containing NaCl, $CaCl_2$ and NaLactate with the concentration 0.514, 0.234 and 0.518 mol/kg of water, respectively. This solution has its ionic strength of 1.102 mol/kg water as calculated by MSA model (**Table 2**), which is similar to that of cheese juice calculated by the DIS model (**Table 1**). The MSA model is developed within McMillan-Mayer (MM) framework on the molarity scale (van Boekel, 2008). The DIS model was developed on molality scale at Lewis-Randall (LR) level. In order to compare the results, conversions were made from MM to LR followed by conversion to activity coefficients on the molarity scale (symbol y) to that on the molality scale (symbol γ) according to the procedure as shown in **Chapter 5**. **Table 2** shows that the Ca^{2+} activity coefficient calculated by the MSA model is about 10% higher than that calculated by the Davies equation. As the ionic strength increases, the difference becomes larger between $\gamma_{Ca,\,MSA}^{LR}$ and $\gamma_{Ca,\,Davies}^{LR}$ (**Table 2**), although both $\gamma_{Ca,\,MSA}^{LR}$ and $\gamma_{Ca,\,Davies}^{LR}$ increase as the ionic strength increases. The excluded volume effects account for the difference. Therefore, it is more suitable to use the MSA model in future to describe ion properties in concentrated systems. However, the DIS model, which currently employs the Davies equation, still

can be used as a first approximation of ion composition in concentrated systems. It would not be a difficult task to incorporate the MSA model in our developed models and it is recommended to do that in future work.

Table 2. Comparison of Ca activity coefficients (y on the molarity scale, γ on the molality scale) calculated by the DIS model and MSA model in one-month old cheddar cheese juice [a].

Ionic strength (mol/kg H_2O)	$y_{Ca,\ MSA}^{MM}$	$y_{Ca,}$	$\gamma_{Ca,}$	$\gamma_{Ca,}$
1.102	0.31	0.29	0.28	0.25
1.404	0.43	0.40	0.37	0.29
1.751	0.61	0.55	0.51	0.36

[a] MSA model is developed to describe the Ca activity coefficient in a solution containing NaCl, $CaCl_2$, and NaLactate with the concentration 0.514, 0.234 and 0.518 mol/kg of water, respectively. This solution is a simplified system of cheese juice, and has an ionic strength of 1.102 mol/kg water.

[b] $\gamma_{Ca,\ Davies}^{LR}$ is the Ca activity coefficient of the cheese juice calculated by the DIS model using the Davies equation.

8.4 Main conclusions and Recommendations

Looking back on the project aims, we conclude our work in a twofold manner:

(i) For a number of milk-based systems under various conditions, the ion compositions have been successfully determined. The Donnan membrane technique (DMT) has been demonstrated to be an accurate method to determine the concentrations of free metal ions in milk-based systems. Calcium ion selective electrode (Ca-ISE) suffers from matrix effects due to the compositional difference between

calibration solutions and milk samples. Standards solutions with compositions similar to milk serum have been developed and the use of them remarkably improves the accuracy of Ca^{2+} activity measurement by Ca-ISE.

(ii) Ion speciation models (EIS, DIS and MIS) give satisfactory descriptions of the ion compositions in milk-based systems for all applied conditions. Disaccharides affect the ion properties through excluded volume effects, which can be successfully explained by the MSA theory. Moreover, the MSA theory is a more suitable tool to describe ion properties in concentrated systems than the Davies equation.

Regarding future research, the limitations of the developed models should be dealt with in order to make the models more robust and complete. Moreover, modelling the effect of temperature on ion equilibria in milk-based systems is another interesting and important topic to focus on. A few points are suggested to achieve this goal. The first is to build an ion speciation model for SMUF as a simplified system of milk at different temperatures. Next, the SMUF model can be extended to describe milk system as was done in this thesis. Experiments need to be carried out to determine the ion compositions in SMUF and milk systems over a range of temperatures. The free metal ion concentrations can be measured by DMT method since Ca-ISE cannot work at high temperatures. The concentrations of ions in milk serum at different temperatures can be determined by using the ultrafiltration method as described by Pouliot et al. (1989). Moreover, the association constants and solubility products of the major ion complexes and solids should be determined at different temperatures. A good example for determination of solubility product is given for $CaHPO_4$ at 90 °C as described by Mooney and

Meisenhelter (1960). The amount of experimental work to be carried out seems impressive, but the present success of our ion speciation models justifies the expectation that they can be developed to a powerful tool for predicting ion compositions in milk-like systems for a broad range of conditions.

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Summary

Ion equilibria of milk, particularly the distribution of calcium phosphate between casein micelles and milk serum, play a crucial role in the conformation and stability of casein micelles. Alterations of ion equilibria can have marked influences on the stability of casein micelles during processing and storage of dairy products, leading to changes in the physicochemical and sensorial properties of the product. The aim of this PhD research was to quantify and model ion composition in terms of concentrations and activities of ions and ion complexes in milk systems. To study the consequences of varying processing steps on ion equilibria, the following conditions were varied: pH, ionic strength (by adding NaCl and KCl), calcium fortification (by adding $CaCl_2$) and calcium complexation (by adding Na_2HPO_4, $Na_4P_2O_7$, $Na_5P_3O_{10}$ and $Na_8P_6O_{19}$), and addition of sugars (sucrose, lactose, maltose and trehalose). All these changes reflect what can happen in various production processes, such as fermentation (pH change), addition of salts in cheese making, complexation agents (in heat-treated concentrated milks and in processed cheese) while sugars are added in dessert products and sweetened condensed milk. The rationale behind this research is discussed in **Chapter 1**.

A prerequisite for modelling ion equilibria is to accurately measure the activities and concentrations of ions, in particular Ca^{2+} and Mg^{2+} ions, which are the major cations and are essential to the structural stability of casein micelles (**Chapter 2**). Some 50 years ago cation exchange techniques and the murexide method were the standard for measuring Ca^{2+} ions; later on the calcium ion selective electrode (Ca-ISE) was developed and became the

standard. Because we wanted another independent method next to ISE, we investigated the potential of the so-called Donnan Membrane Technique (DMT), developed for soil systems. DMT was successfully applied to determine free Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} ions in milk systems. Specific characteristics of DMT are: (1) simultaneous determination of various free metal ions; (2) minimal distortion of sample equilibrium; (3) minimal interferences from other components. Moreover, DMT might be suitable to measure free Ca^{2+} ion concentration at elevated temperatures (up to 90 °C), which is important to understand ion speciation at high temperature for milk products (but this was not investigated in the present study).

To model ion speciation in milk, a simplified system named simulated milk ultrafiltrate (SMUF) was studied first (**Chapter 3 and 4**). SMUF is a solution to mimic mineral composition of milk serum, without the presence of casein micelles, which contain colloidal calcium phosphate and organic phosphoserine calcium complexes. SMUF has been proposed as a model system since 50 years and is used very often in dairy research. It appeared, however, that freshly prepared SMUF is a solution that is supersaturated with respect to various calcium phosphate phases, and hence is not in equilibrium. A significant difference in ionic composition appeared to exist between freshly prepared SMUF and SMUF that has reached equilibrium. Two ion speciation models were developed to describe these two situations: the EIS model (Equilibrium Ion Speciation, describing the ion composition for SMUF at equilibrium) and the DIS model (Dynamic Ion Speciation, describing the ion composition of freshly prepared SMUF). Both models deal with the major ions, ion complexes and solid precipitates formed in SMUF. The mass balance equation for each component was constructed and a group of intrinsic association constants and solubility products were employed,

partly taken from literature and partly estimated. The resulting equations describing electrolyte equilibria (called ion speciation) are non-linear algebraic equations that were solved numerically to obtain activities and concentrations for all the components and their complexes. A dedicated commercially available software program was used for the calculations (AESolve).

Both of the models were validated by experiments in which $CaCl_2$ and Na_2HPO_4 were added to freshly prepared SMUF solution. The EIS model gave predictions in ion concentrations and activities, pH and solids precipitation at equilibrium for SMUF, which were in line with the experimental results. The precipitates formed were investigated by X-ray diffraction and the outcomes were in line with the composition predicted by the ion speciation model. The DIS model was able to predict the ion composition of fresh SMUF and SMUF enriched with $CaCl_2$ or Na_2HPO_4 at the initial stage; the difference with the EIS model is that empirical kinetics was added to account for the metastable situation in SMUF. Additional conditions, e.g. pH, ionic strength and calcium chelating agents (various polyphosphates) were used and the modelling results were in general agreement with experimental data. Moreover, sodium ions were found not to be equivalent to potassium ions since they resulted in different Ca^{2+} and Mg^{2+} concentrations and activities, and pH in SMUF. This could be well explained by the difference in their hydration properties. In addition, the developed model suggests that sodium hexametaphosphate ($Na_8P_6O_{19}$) displays chain polyphosphate properties (not a ring structure as is proposed in literature), which provides better understanding of its interaction with cations.

As a next step, it was studied how addition of non-electrolytes such as sugars

would influence ion properties (**Chapter 5**). Sugars, as non-electrolytes, were found to increase Ca^{2+} ion activities and decrease pH in aqueous solutions ($CaCl_2$-KCl-K_3Citrate solution, SMUF) and milk if sugars were added to these systems. The so-called mean spherical approximation (MSA) theory was used to explain the impact of sugars on ionic properties in milk systems by taking into account electrostatic interactions and volume exclusion effect. The effect of sucrose on ionic properties in an electrolyte solution consisting of $CaCl_2$, KCl and K_3Citrate was calculated using a MSA model. It appeared that the excluded volume effect accounted for a considerable increase in activity coefficient of the ions, implying that the activity increases even when the concentration remains the same. This offered a sufficient explanation of the observed increase in Ca^{2+} activity and the decrease in pH in milk systems enriched with sugars. Addition of various sugars (sucrose, maltose, lactose, trehalose) resulted in similar thermodynamic properties of milk-like systems, in line with the suggestion that the observed effect is due to a non-specific volume exclusion effect.

As a final step, a model describing ion speciation in milk was developed based on the EIS and DIS models (**Chapter 6**). This Milk Ion Speciation (MIS) model includes not only all the components of SMUF, but also colloidal calcium phosphate (CCP) and phosphoserine calcium complexes of casein micelles. The nature of CCP at pH 6.70 was considered to be basic since the molar ratio of Ca/P_i was measured as 1.84. The model suggests that the composition of CCP is a mixture of a basic calcium phosphate ($Ca_4(PO_4)_2(OH)_2$) and an acidic calcium phosphate ($CaHPO_4.2H_2O$). Moreover, the composition of CCP may change as a function of pH. Experiments were carried out to determine the ionic properties of milk under varying conditions, such as pH, ionic strength (NaCl or KCl), $CaCl_2$, calcium

chelating agents (Na_2HPO_4, and $Na_4P_2O_7$). The model predictions were in general agreement with experimental results for all conditions (within 15% deviation).

From the many measurements of Ca^{2+} activity measurement during this PhD study, we observed a significant difference in Ca^{2+} activity measurement between Ca-ISE method and DMT technique at lower ionic strength ($I < 0.1$ mol/kg water). It was shown in **Chapter 7** that the ionic compositional difference between conventional calibration solutions and milk-based samples had a significant effect on the single Ca^{2+} activity coefficient, which generates erroneous estimates of Ca^{2+} activities in SMUF and milk. To overcome this problem, we developed new calibration standards which have similar compositions to milk serum by means of the DIS model. Consequently, the new standards showed a significant improvement in the accuracy of Ca^{2+} activity and Ca^{2+} activity coefficient over the conventional standards. After having implemented this correction, the difference in Ca^{2+} activity between the Ca-ISE method and DMT technique was within experimental error.

Finally, the ion speciation models (EIS, DIS and MIS) were carefully evaluated and a case study of applying the developed ion speciation models to cheese was carried out to challenge and test the predictive capability of the models, as described in the general discussion (**Chapter 8**). Particularly, the nature of colloidal calcium phosphate (CCP) was intensively discussed. We suggest that CCP is a mixture of calcium phosphates with acidic form ($CaHPO_4$) and basic form ($Ca_4(PO_4)_2(OH)_2$, HAP-like) together with $MgHPO_4$ and $Ca_3citrate_2$. Moreover, calculation of the ion composition in skim milk enriched with polyphosphate (TSPP) was attempted. Unfortunately,

the model calculation did not give satisfactory predictions in pH, concentrations of serum P_i and Ca in this case, for which we cannot yet explain this discrepancy. Another important trial was to use the models to calculate ion equilibria in cheese juice. The models were demonstrated to give satisfactory predictions of ion composition in concentrated systems (i.e., cheese juice). The message is, therefore, that the developed models offer great potential and can be a powerful tool to correlate behaviour of ions to physicochemical, microbial and sensorial properties of cheese. The limitations of the developed models have been discussed and should be dealt with in order to make the models even more powerful tools to describe ion composition in milk for a broad range of conditions.

In conclusion, the developed models appear to be robust and versatile tools to describe ion speciation in milk-like systems. Based on this approach, a significant step can be made in transferring fundamental knowledge to the R&D environments of the dairy industry in particular, but also to that of the food industry in general. There this knowledge can then be applied in new product development and also can be used to deliver solutions to problems in existing products.

Samenvatting

Ion-evenwichten in melk spelen een belangrijke rol in de conformatie en stabiliteit van caseinemicellen, met name de verdeling van calciumfosfaat over caseïnemicellen en melkserum,. Veranderingen in ion-evenwichten tijdens bewerking en opslag kunnen van grote invloed zijn op de stabiliteit van caseinemicellen in zuivelproducten en daarmee op de fysisch-chemische en sensorische eigenschappen van de producten.

Het doel van het onderzoek beschreven in dit proefschrift was om de ionsamenstelling in melk en daarvan afgeleide systemen te kwantificeren en te modelleren d.m.v. wiskundige modellen in termen van concentraties en activiteiten van ionen en ioncomplexen. De gevolgen van verschillende bewerkingsstappen op ion-evenwichten werden bestudeerd door de volgende condities te varieren: pH, ionsterkte (door NaCl and KCl toe te voegen), $CaCl_2$ toevoeging, het toevoegen van calciumbinders als Na_2HPO_4, $Na_4P_2O_7$, $Na_5P_3O_{10}$ and $Na_8P_6O_{19}$, en het toevoegen van suikers (sacharose, lactose, maltose en trehalose). Deze veranderingen weerspiegelen wat er kan gebeuren in allerlei bewerkingen, zoals fermentatie (leidend tot pH verandering), toevoegen van zouten bij de kaasbereiding, het toevoegen van calciumbinders aan te verhitten geconcentreerde melk en smeerkaas, terwijl suikers worden toegevoegd aan allerlei dessert producten en gesuikerde condens. De achtergrond, de verantwoording en de opzet voor het onderzoek is beschreven in **Hoofdstuk 1**.

Een voorvereiste om ion-evenwichten te kunnen modelleren is het nauwkeurig kunnen meten van activiteiten en concentraties van ionen, met

name de tweewaardige ionen Ca^{2+} and Mg^{2+}, de voornaamste kationen die essentieel zijn voor de structuur en de stabiliteit van caseinemicellen (**Hoofdstuk 2**). Zo'n 50 jaar geleden waren technieken als ionenuitwisseling en de murexide methode de standaard voor het meten van Ca^{2+} ionen; ongeveer 10 jaar daarna kwam de ion-selectieve calcium electrode ter beschikking (Ca-ISE) en werd deze de standaard. Niettemin werd het in dit onderzoek nodig bevonden om de beschikking te hebben over nog een andere onafhankelijke methode en die is gevonden in de zogenaamde Donnan Membraan Techniek (DMT), in eerste instantie ontwikkeld voor het bestuderen van ionevenwichten in de bodem. DMT bleek een uitstekende methode om vrije Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} ionen in melksystemen te bepalen. Enkele specifieke karakteristieken van DMT zijn: (1) het simultaan kunnen bepalen van verschillende vrije metaal ionen; (2) een minimale verstoring van de ion-evenwichten tijdens de bepaling; (3) minimale interferentie van de andere aanwezige componenten op de analyse. Bovendien zou DMT het mogelijk moeten maken om de vrije Ca^{2+} ion concentratie te meten bij verhoogde temperatuur (tot 90 °C), hetgeen van belang is om het gedrag van ionevenwichten en hun associaties te begrijpen in verhitte melk producten, maar dit is niet bestudeerd in dit proefschrift.

Het mathematisch modelleren van iongedrag werd eerst bestudeerd in een systeem dat de ion-samenstelling van melkserum imiteert, in het Engels genaamd *simulated milk ultrafiltrate* (SMUF). Het onderzoek aan dit systeem wordt beschreven in **Hoofdstukken 3 and 4**. SMUF is dus een waterige oplossing zonder caseinemicellen en colloïdaal calciumfosfaat. SMUF wordt al 50 jaar intensief gebruikt als modelsysteem in het zuivelonderzoek. Het bleek echter dat een vers bereide SMUF oplossing niet in evenwicht is en oververzadigd is t.a.v. verschillende vormen van calciumfosfaat. Als men een

SMUF oplossing de tijd geeft om wel evenwicht te bereiken ontstaan precipitaten en er blijkt dan een aanzienlijk verschil te bestaan in ionsamenstelling tussen vers bereid SMUF en tot evenwicht gekomen SMUF. Daarom zijn er uiteindelijk twee mathematische modellen ontwikkeld: het EIS model (*Equilibrium Ion Speciation*) dat de ionsamenstelling beschrijft van SMUF in evenwicht, en het DIS model (*Dynamic Ion Speciation*) dat de ionsamenstelling van vers bereid SMUF beschrijft maar ook beschrijft hoe de ionevenwichten veranderen als het systeem op weg is naar evenwicht. Beide modellen beschrijven de voornaamste ionen, ioncomplexen en precipitaten in SMUF. De modellen bestaan uit wiskundige vergelijkingen die een massabalans voor elke component beschrijven, alsmede de verschillende dissociaties en associaties voor de ionevenwichten, gekarakteriseerd door intrinsieke associatie constanten en oplosbaarheidsproducten. Deze parameters werden gedeeltelijk uit de literatuur gehaald en gedeeltelijk zelf geschat. De resulterende vergelijkingen zijn niet-lineaire algebraïsche vergelijkingen die numeriek werden opgelost om aldus activiteiten en concentraties voor alle componenten en hun complexen te kunnen berekenen. Dit werd gedaan met een daartoe speciaal (commercieel beschikbaar) software programma, AESolve geheten.

Beide modellen werden gevalideerd door middel van experimenten waarin $CaCl_2$ en Na_2HPO_4 werden toegevoegd aan vers bereide SMUF oplossingen. Het EIS model voorspelde aldus ion concentraties en activiteiten, de pH en gevormde precipitaten voor SMUF in evenwicht en de voorspellingen waren in overeenkomst met experimentele resultaten. De gevormde precipitaten werden verder onderzocht d.m.v. *X-ray* diffractie en de uitkomsten kwamen overeen met de door het EIS model voorspelde samenstelling. Het DIS model bleek in staat om de ionsamenstelling te voorspellen van vers bereid SMUF

en ook van SMUF waaraan $CaCl_2$ of Na_2HPO_4 was toegevoegd. Het verschil met het EIS model is dat het DIS model rekening moet houden met een metastabiel systeem; dit werd bereikt door een empirische kinetische vergelijking toe te voegen die de tijdsafhankelijke precipitatie beschreef en de daarmee samenhangende veranderingen in ionevenwichten. Experimentele condities werden gevarieerd d.m.v. pH verandering, verandering van ion sterkte, en toevoeging van calcium-bindende agentia (polyfosfaten); de door het model voorspelde resultaten kwamen in het algemeen goed overeen met de experimentele resultaten. Verder kwam duidelijk naar voren dat natrium ionen zich niet hetzelfde gedragen als kalium ionen omdat het toevoegen van deze ionen aan SMUF tot verschillende Ca^{2+} and Mg^{2+} concentraties en activiteiten en pH bleek te leiden. Dit werd verklaard uit het verschil in interactie van deze ionen met water. Ook voorspelt het ontwikkelde model dat natrium hexametafosfaat ($Na_8P_6O_{19}$) zich als een keten gedraagt en niet als een ring zoals in de literatuur wordt gesuggereerd. Deze bevinding gaf een beter inzicht in de interactie van $Na_8P_6O_{19}$ met kationen.

De volgende stap was om te bestuderen of en hoe suikers, als ongeladen moleculen, invloed kunnen hebben op het gedrag van ionen (**Hoofdstuk 5**). Het bleek dat suikers de Ca^{2+} ion activiteit verhogen en de pH verlagen in waterige zout oplossingen als $CaCl_2$-KCl-K_3Citraat en SMUF. Om dit onverwachte effect te verklaren werd de zogenaamde *mean spherical approximation* (MSA) theorie toegepast op de gebruikte systemen. Deze theorie brengt zowel electrostatische interacties van geladen moleculen als volume-uitsluiting van geladen én ongeladen moleculen in rekening. Berekeningen met sacharose in $CaCl_2$-KCl-K_3Citraat oplossing toonden aan dat volume-uitsluiting door sacharose resulteert in aanzienlijke verhoging van de activiteitscoefficient van ionen, hetgeen betekent dat bij gelijkblijvende

concentratie de activiteit toch toeneemt, hetgeen experimenteel ook werd waargenomen. De modelberekeningen toonden aan dat de waargenomen effecten ook kwantitatief konden worden verklaard. Het toevoegen van verschillende soorten suikers (sacharose, maltose, lactose, trehalose) resulteerde in dezelfde thermodynamische eigenschappen, hetgeen als een bevestiging gezien kan worden dat het hier gaat om niet-specifieke volume-uitsluiting effecten.

Op basis van de hierboven genoemde modellen EIS en DIS werd een wiskundig model ontwikkeld om het gedrag van ionen in melk te kunnen beschrijven, het zogenaamde *Milk Ion Speciation* (MIS) model (**Hoofdstuk 6**). Naast alle SMUF componenten werden hier nu ook colloïdaal calciumfosfaat (CCP) en calcium-serinefosfaat complexen (beide aanwezig in caseïnemicellen) in meegenomen. De aard van CCP bij pH 6.70 werd beschouwd als basisch vanwege de gemeten molaire verhouding van Ca/P_i als 1.84. Uit het model werd afgeleid dat de samenstelling van CCP beschouwd kan worden als een mengsel van een basisch calciumfosfaat ($Ca_4(PO_4)_2(OH)_2$) en een zuur calciumfosfaat ($CaHPO_4.2H_2O$), maar ook dat deze samenstelling verandert als functie van de pH. Experimenten werden uitgevoerd om de veranderingen in iongedrag te bestuderen als functie van variërende omstandigheden zoals pH, ionsterkte (bewerkstelligd door het toevoegen van NaCl of KCl), $CaCl_2$ toevoeging, calcium bindende agentia (Na_2HPO_4, $Na_4P_2O_7$). De model voorspellingen kwamen in het algemeen goed overeen met de waargenomen resultaten (binnen 15%), hetgeen gezien kan worden als een validatie van het MIS model.

Uit de vele Ca^{2+} activiteit metingen verricht in dit onderzoek kwam een significant verschil naar voren in Ca^{2+} activiteit metingen tussen de Ca-ISE

methode en de DMT techniek bij lage ionsterkte (I < 0.1 mol/kg water). In **Hoofdstuk 7** wordt beschreven dat dit te wijten is aan verschillen in ionsamenstelling tussen de aanbevolen simpele ijkoplossingen (bestaande uit $CaCl_2$ en NaCl) en de te meten oplossingen (melk-achtige systemen in dit geval), leidend tot een significant effect op de Ca^{2+} activiteits coefficient en bijgevolg tot fouten in Ca^{2+} activiteitsmetingen in SMUF en melk. Dit probleem verdwijnt als nieuwe ijkoplossingen worden gebruikt die een vergelijkbare ionsamenstelling hebben als de te meten oplossingen. Deze nieuwe ijkoplossingen werden samengesteld op basis van het DIS model. Door deze correcties verdwenen de verschillen in Ca^{2+} activiteit tussen de Ca-ISE methode en de DMT techniek binnen de experimentele meetfout.

In de algemene discussie (**Hoofdstuk 8**) werden de ontwikkelde modellen EIS, DIS en MIS kritisch geëvalueerd en op hun waarde geschat. Bovendien werden de modellen getest op systemen die niet waren gebruikt bij het ontwikkelen van de modellen. In het bijzonder werd aandacht besteed aan de aard van het colloïdale calciumfosfaat (CCP). Geconcludeerd werd dat het calciumfosfaat in CCP als een mengsel beschouwd kan worden van een zure vorm ($CaHPO_4$) en een basische vorm ($Ca_4(PO_4)_2(OH)_2$, lijkend op hydroxyapatiet, HAP) terwijl daarnaast ook $MgHPO_4$ and $Ca_3Citraat_2$ aanwezig zijn. Ook werd getracht de ionsamenstelling te berekenen van ondermelk waaraan polyfosfaat (TSPP) was toegevoegd. Hier bleef het model deels in gebreke omdat geen bevredigende voorspelling van de pH en concentraties van fosfaat en calcium in het serum werd gevonden. Er kon geen verklaring worden geboden voor deze discrepantie. De beperkingen van de modellen zijn bediscussieerd en suggesties zijn gegeven om de modellen verder te ontwikkelen voor een breed scala aan condities.

Verder werden de modellen nog ingezet om de ionsamenstelling in de waterige fase van kaas te berekenen en de berekeningen kwamen goed overeen met de experimentele resultaten. Dit werd als een belangrijk resultaat beschouwd omdat dat betekent dat de modellen ook voorspellende waarde hebben voor systemen waarvoor de modellen in eerste instantie niet waren ontwikkeld.

Als algemene conclusie uit dit werk kan gesteld worden dat de ontwikkelde modellen behoorlijk robuust blijken en flexibel inzetbaar blijken te zijn. De modellen zijn gebaseerd op heel fundamentele wetenschappelijke kennis en gewapend met deze kennis blijkt dat daarmee ook hele praktische problemen kunnen worden benaderd op een kwantitatieve manier. Er kan dus een significante stap worden gemaakt van fundamenteel naar toegepast onderzoek in R&D omgevingen van de levensmiddelenindustrie en de zuivelindustrie in het bijzonder. De modellen zijn bijzonder krachtige hulpmiddelen en productontwikkeling kan aldus rationeler worden gedaan bij het ontwikkelen van nieuwe producten maar ook in het verbeteren van bestaande producten.

Acknowledgements

Wageningen, a small town in the Netherlands, has become my second home in my life. I had an amazing and unforgettable time in the past seven years with my family, friends, colleagues, and supervisors who have been supporting and encouraging me to accomplish my PhD work. It is my great honour to have worked with all of you.

I would like to express my special thank to Prof. Tiny van Boekel. I still remember that I expressed my wish to talk to you more regularly in my first year R&O meeting. In the last three years, we discussed a lot and solved many difficult problems. The most important thing is that I learned to work structurally and to be critical from you. These fruitful results are due to your delicate and patient guidance. I wish that I still can discuss scientific work in the future with you.

I also want to thank my co-supervisor Prof. Herman van Leeuwen. Thank you very much for your great interest in dairy systems and dedicated efforts to teach me the fundamentals of electrochemistry. I will never forget the texts, equations and drawings on the whiteboard in your office.

I also want to express my gratitude to my daily supervisor Hein van Valenberg for his great support and care in my PhD life. Hein, we know each other for seven years now. I am your first Chinese student, and you are the person who gave me the opportunity to do an internship at Campina and to help me to be a PhD student at PDQ. Moreover, you taught me how to make a decision, how to manage my project and how to overcome my weakness.

Acknowledgements

I would like to thank Matthias Eisner, Stacy Pyett, Jurriaan Schlatmann for initiating, managing and supervising this PhD project. All of you brought me to know the company environment and gave me opportunities to work on company projects. I gained lots of benefits from these experiences, which led to great success of my PhD project. I also want to thank William Kloek and Maarten van de Ven for their advice to my project.

Next, I want to express my appreciation to Erwin Temminghoff for his magnificent contribution in helping me to set up the Donnan Membrane Technique. Erwin, your valuable advice on the Donnan Membrane Technique did guarantee that the whole project was running successfully.

Another special thank is given to Prof. Toon van Hooijdonk for offering critical and valuable viewpoints to my PhD project.

Part of the PhD work has been carried out by my MSc students: Xiao Liu, Radhika Bongoni, Faridah, Meillianti, and Banu Dal. I really appreciate your accurate work. Without your dedicated efforts and hard work, this PhD work could not have beeen so successful and promising.

I also would like to thank all the people from the Product Design and Quality Management Group and Food Physics Group. Particularly, I had a great time with colleagues in room 211B. A special thank to Mirko Bollen, Jeroen Knol, Jeroen Heck and Kasper Hettinga. There are lots of great memories in the past 4 years with you guys. Wherever we are in the future, I hope that our friendship lasts forever. I also want to thank Etske, Lu, Muhammad, Van-Anh, Kristin, Jenneke, Teresa, Yann, Francisco for your great support. In addition, I am very grateful to Frans Lettink for his help to my experimental work.

Furthermore, I would like to express my gratitude to my Chinese friends: Nie Haisheng, Liu Huaifeng, Huang Huiyan, Xiao Yinghua, Qin Yin, Tian Lijin, Li Zhaoying, Lu Jing, Li Yuan, Cheng Xu, Ru Yu, Wang Si, Yu Li, Yi Liya, Wei Jianing, Liang Jianfen, Prof. Han Beizhong, Prof. Chen Hongbing in Wageningen for your support. In particular, a special thank is given to Dr.Nie Haisheng for his endless support. It is hard to express my gratefulness in words to you. May our solid friendship keep for the whole life. Another special thank is to Liu Huaifeng and Huang Huiyan. We had good times between our families in the past 4 years. I really cherish the friendship with both of you. All the best to your life in the Netherlands and bright future in China. In addition, I want to say an extra thank to Lu Jing for being my paranimf. It is my great pleasure to work with you.

最后，我要对父亲母亲及妻子表达衷心的谢意。感谢爸妈日夜辛劳给我创造的留学条件;在过去的七年里，您们一直在遥远的祖国鼓舞着我; 没有您们的支持, 我是不可能有今天的成就。罗婧, 有了你的陪伴，我体会到生活是如此的精彩；有了你的支持，无论多少困难，我都能够一往无前；我期待着我们在新西兰的生活会更精彩。这本博士论文是献给你们最好的礼物。

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2010-08-01

Curriculum vitae

Gao Ran (高然) was born on Jan 19th 1981 in Hebei Province of China. In the year between 1999 and 2003, he completed his Bachelor study in the College of Food Science and Nutritional Engineering in China Agricultural University in Beijing China. After that, he started MSc study and majored in Food Technology-Product Functionality in Wageningen University in the Netherlands. He performed his MSc thesis in the Laboratory of Food Chemistry in Wageningen University from August 2004 to March 2005. Subsequently, he worked as a trainee in Campina Innovation to investigate the factors influencing protein gelation at sweet/sour barrier of dairy desserts under supervision of Dr. Stacy Pyett and Dr. William Kloek. He graduated in September 2005 from Wageningen University, and returned to China to have a temporary job as a product supervisor in the company Xin Jiang Tomato-red biotechnology Co. Ltd in Beijing. From February 2006 to May 2010, he worked as a PhD student on the PhD project named “Ion Speciation in Milk-like Systems” in Product Design and Quality Management Group in Wageningen University. Since June 1st 2010, he started to work as a Research Technologist in Fonterra Research Centre in New Zealand.

List of publications

Research articles

Gao, R., van Leeuwen, H. P., van Valenberg, H. J. F., Eisner, M. D., & van Boekel, M. A. J. S. Quantification and calculation of the ion composition in reconstituted skim milk and the consequences of adding electrolytes: HCl, NaCl, KCl, $CaCl_2$ and Na_2HPO_4 (submitted for publication).

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Gao, R., Eisner, M. D., & van Boekel, M. A. J. S. (2009). Modelling the ion composition in simulated milk ultrafiltrate under the influence of pH, ionic strength and calcium chelating agents. *European Dairy Magazine*, 12-15.

Overview of completed training activities

Discipline specific activities

Courses and conferences

VLAG Reaction Kinetics in Food Science, 2006, Wageningen, The Netherlands

VLAG Industrial Proteins, 2006, Wageningen, The Netherlands

OSPT A Unified Approach to Mass Transfer, 2007, Wageningen, The Netherlands

VLAG Polysaccharide as Food Colloids and Biomaterials, 2007, Wageningen, The Netherlands

Han-sur-Lesse Winter school TU Delft, 2009, Han-sur-Lesse, Belgium

Alltech Chromatography Symposium, 2005, Wageningen, The Netherlands

28th World Dairy Summit, 2007, Dublin, Ireland

1st International Symposium on Minerals and Dairy Products, 2008, Saint-Malo, France

Milk conference 2009, BOKU university, Wien, Austria

6th NIZO Dairy Conference, 2009, Papendal, The Netherlands

General courses

PhD Competence Assessment, 2006, WUR

Academic Writing II, 2006, WUR

Information Literacy for PhD, 2007, WUR

EndNote Basic and Advanced, 2007, WUR

Scientific Writing, 2008, WUR

Digital Artwork and Photoshop in Life Sciences, 2008, WUR

Project and Time Management, 2009, WUR

Career Orientation, 2009, WUR

Career Assessment, 2009, WUR

PhD excursions

16th VLAG PhD week, 2006, Emerlo, The Netherlands

PhD study trip of Product Design and Quality Management Group, 2007, USA

PhD excursion of Dairy Technology Cluster, 2009, University of Ghent, Belgium

The research described in this thesis was financially supported by FrieslandCampina Innovation Europe.

Wöhrmann Print Service is gratefully acknowledged for printing this thesis.